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(54) Title: HUMAN GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMINO-TRANSFERASE

(57) Abstract

Isolated DNA molecules encoding human glutamine:fructose-6-phosphate amidotransferase are provided. The DNA molecules are useful within methods to screen for glutamine:fructose-6-phosphate amidotransferase antagonists. Briefly, DNA molecules encoding human glutamine:fructose-6-phosphate amidotransferase are expressed in suitable host cells, and recombinant glutamine:fructose-6-phosphate amidotransferase is produced. A test substance is exposed to the recombinant human glutamine:fructose-6-phosphate amidotransferase in the presence of fructose-6-phosphate and glutamine. A reduction in activity of the glutamine:fructose-6-phosphate amidotransferase in comparison to the activity in the absence of the test substance indicates a compound which inhibits human glutamine:fructose-6-phosphate amidotransferase.

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HUMAN GLUTAMINE:FRUCTOSE-6-PHOSPHATE AMINO-TRANSFERASE

Background of the Invention

The glucoregulatory effects of insulin are predominantly exerted in the liver, skeletal muscle and adipose tissue. Insulin binds to its cellular receptor in these three tissues and initiates tissue-specific actions that result in, for example, the inhibition of glucose production and the stimulation of glucose utilization. In the liver, insulin stimulates glucose uptake and inhibits gluconeogenesis and glycogenolysis. In skeletal muscle and adipose tissue, insulin acts to stimulate the uptake, storage and utilization of glucose.

In certain disease states, the cellular mechanisms that result in the overall regulation of blood glucose levels are impaired, resulting in abnormally high levels of circulating insulin and glucose. Type II non-insulin dependent diabetes (also known as NIDDM and referred to hereinafter as Type II diabetes), which involves pancreatic islet cell dysfunction and insulin resistance, is characterized by abnormal basal and stimulated insulin secretion, increased endogenous hepatic glucose release and inefficient peripheral tissue glucose utilization, resulting in hyperinsulinemia and hyperglycemia. Insulin resistance has also been documented in obesity, pregnancy, acromegaly, hypertension, atherosclerosis and certain catabolic situations associated with glucose intolerance.

While insulin receptor mutations and decreased numbers of insulin receptors have been reported in patients exhibiting insulin resistance, such abnormalities in themselves do not explain the overall inability of insulin to stimulate glucose uptake in peripheral tissues such as in skeletal muscle and adipose tissue. A decrease in insulin-stimulated glucose uptake has been observed in adipocytes from obese and/or Type II

diabetic patients even at maximally-effective insulin concentrations (for review, see Olefsky and Molina in Diabetes Mellitus Theory and Practice, Fourth Edition, pp 121-153, Rifkin and Porte, eds, New York, NY, 1990).

Cushman and Wardzala (J. Biol. Chem. 255: 4758-4762, 1980) proposed that insulin stimulates glucose transport into isolated adipocytes through a rapid, reversible, energy-dependent process culminating with the translocation of glucose transporters from an intracellular pool to the plasma membrane. Subsequent studies demonstrated that the number of intracellular glucose transporters was reduced in insulin resistant states such as in streptozotocin-induced diabetes, obesity and high fat feeding (Karnieli et al., J. Clin. Invest. 68: 811-814, 1981; Hissin et al., J. Clin. Invest. 70: 780-790, 1982; and Hissin et al., Diabetes 32: 319-325, 1983). Studies by Traxinger and Marshall (J. Biol. Chem. 264: 20910-20916, 1989) demonstrated that desensitization of the glucose transport system in primary rat adipocyte cultures could be achieved with a combination of insulin, glucose and glutamine. The demonstration that the glutamine analogs O-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-norleucine, which irreversibly inactivate glutamine-requiring enzymes, inhibit the desensitization of the insulin-responsive glucose transport system in primary rat adipocyte cultures led Marshall et al. (J. Biol. Chem. 266: 4706-4712, 1991) to suggest that the utilization of glucose through the hexosamine pathway plays a key role in the insulin-mediated glucose transport system. Additionally, Marshall et al. (ibid., 1991) showed that treatment of primary rat adipocyte cultures with glucosamine effectively induces insulin resistance. Glucosamine enters the hexosamine pathway as glucosamine-6-phosphate, which is the enzymatic product of glutamine:fructose-6-phosphate amidotransferase (referred to hereinafter as GFAT).

Studies of GFAT activity in desensitized rat adipocytes led Traxinger and Marshall (J. Biol. Chem. 266: 10148-10154, 1991) to suggest that GFAT activity is regulated in a coordinated manner by insulin, glucose and glutamine. Marshall et al. (J. Biol. Chem. 266: 10155-10161, 1991) proposed a model in which increased metabolism of glucose through the hexosamine pathway, and more particularly through the GFAT step of the pathway, leads to an intracellular signal that inhibits the recruitment and activation of glucose transporters by insulin which in turn leads to insulin resistance. Marshall et al. (J. Biol. Chem. 266: 10155-10161, 1991) suggest a role for GFAT in pathophysiological insulin-resistant states such as Type II diabetes. GFAT coding sequences have been isolated from Saccharomyces cerevisiae (Watzele and Tanner, J. Biol. Chem. 264: 8753-8758, 1989), E. coli (Walker et al., Biochem. J. 224: 799-815, 1984; and Badet et al., Biochemistry 26: 1940-1948, 1987) and R. leguminosarum (Surin and Downie, Mol. Microbiol. 2: 173-183, 1988), however, the human coding sequence has not been elucidated.

For patients with Type II diabetes, treatment regimes include body weight reduction, insulin administration and oral sulfonylureas. Caloric restriction to achieve body weight reduction decreases the overall caloric intake and, more specifically, decreases glucose intake and reduces hepatic glycogen stores. Maintenance of a lower body weight results in a lower plasma glucose level. Patients that derive the most benefit from body weight reduction are those with poor islet function and marked hyperglycemia. Exogenous insulin administration serves to substitute for defective insulin secretion from islet cells. Exogenous insulin serves to correct a hypoinsulinemic condition and relies on the ability of insulin to suppress hepatic glucose release and enhance peripheral glucose uptake. Sulfonylurea administration serves to enhance insulin

secretion and functions in the same way as exogenous insulin administration to lower plasma glucose levels.

The current treatment regimes attempt to overcome the hyperglycemia present in Type II diabetics by boosting circulating insulin to achieve a maximal insulin-stimulated glucose uptake; however, none of the currently available therapeutics can fully overcome the insulin resistant state. There is therefore a need in the art for a method for detecting therapeutic compounds capable of inhibiting insulin resistance.

The present invention fulfills this need by providing materials and methods for use in detecting compounds capable of inhibiting GFAT activity and for preparing oligonucleotide probes capable of detecting glutamine:fructose-6-phosphate amidotransferase sequences. The invention provides for the identification of compounds that inhibit GFAT activity through the use of an assay system employing recombinant human GFAT. Such compounds are useful, for example, in inhibiting endogenous GFAT activity and thereby inhibiting insulin resistance.

Summary of the Invention

Briefly stated, the present invention discloses isolated human glutamine:fructose-6-phosphate amidotransferase (GFAT) and isolated DNA molecules encoding human GFAT. In one embodiment of the invention, a representative human GFAT comprises the amino acid sequence of Sequence ID NO: 2 from methioinine, amino acid number 1, to glutamic acid, amino acid number 681. In another embodiment of the invention, representative DNA molecules encoding GFAT include the DNA sequence which comprises the nucleotide sequence shown in Sequence ID NO: 1 from nucleotide 123 to nucleotide 2165. Within another embodiment of the invention, representative DNA molecules encoding GFAT encode the amino acid sequence of

Sequence ID NO: 2 from methionine, amino acid number 1, to glutamic acid, amino acid number 681.

Within yet another embodiment of the invention, an antiserum is obtained from an animal immunized with the human GFAT wherein the antiserum binds to human GFAT. In another embodiment of the invention, a monoclonal antibody against human GFAT is obtained.

Within other embodiments of the invention, DNA molecules of at least about 14 nucleotides are disclosed wherein the molecules are capable of hybridizing with a gene which encodes a human GFAT polypeptide and wherein the DNA molecule is at least 85% homologous to a corresponding DNA sequence of the human GFAT shown in Sequence ID NO: 1 or its complement. In other embodiments of the invention, the DNA molecules of at least about 14 nucleotides are labeled to provide a detectable signal.

In certain embodiments of the invention, DNA constructs containing the information necessary to direct the expression of GFAT are disclosed. Within other embodiments of the invention, host cells containing DNA constructs containing information necessary for the expression of GFAT are disclosed. In certain embodiments of the invention, methods for producing recombinant human GFAT are disclosed. Within other embodiments, recombinant human GFAT is produced from cultured mammalian, bacterial or fungal cells according to the disclosed methods.

In certain embodiments of the invention, methods for detecting a compound which inhibits human GFAT are disclosed. Within the methods, a test substance is exposed to human GFAT in the presence of fructose-6-phosphate and glutamine under physiological conditions and for a time sufficient to allow the test substance to inhibit GFAT activity, wherein a reduction in activity of the GFAT in comparison to the activity in the absence of the test substance is indicative of the presence in the

test substance of a compound which inhibits human GFAT. Within a preferred embodiment, compounds which inhibit human GFAT are detected by measuring the production of radiolabeled glutamate in the presence of a test substance relative to the production of radiolabeled glutamate in the absence of the test substance. Within yet another preferred embodiment, a test substance is exposed to human GFAT in the presence of 3-acetylpyridine adenine dinucleotide, glutamate dehydrogenase, fructose-6-phosphate and glutamine, and the 3-acetylpyridine adenine dinucleotide production is measured relative to the production of 3-acetylpyridine adenine dinucleotide in the absence of the test substance.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA construct: A DNA molecule, or a clone of such a molecule, which has been constructed through human intervention to contain sequences arranged in a way that would not otherwise occur in nature.

Expression vectors are DNA constructs which contain, inter alia, a DNA sequence encoding a protein of interest together with a promoter and other sequences, such as a transcription terminator and polyadenylation signal, that facilitate expression of the protein. Expression vectors further contain genetic information that provides for their replication in a host cell, either by autonomous replication or by integration into the host genome. Examples of expression vectors commonly used for recombinant DNA are plasmids and certain viruses, although they may contain elements of both. They also may include one or more selectable markers.

Transfection or transformation: The process of stably and hereditably altering the genotype of a recipient cell or microorganism by the introduction of

isolated DNA. This is typically detected by a change in the phenotype of the recipient organism. The term "transformation" is generally applied to microorganisms, while "transfection" is generally used to describe this process in cells derived from multicellular organisms.

An object of the present invention is to provide methods for detecting GFAT inhibitors. A feature of the present invention is an isolated DNA molecule encoding human GFAT. Such molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are naturally associated and may include naturally occurring 5' and 3' untranslated sequences that represent regulatory regions such as promoters and terminators. The identification of regulatory regions within the naturally occuring 5' and 3' untranslated regions will be evident to one of ordinary skill in the art (for review, see Dynan and Tijan, Nature 316: 774-778, 1985; Birnstiel et al., Cell 41: 349-359, 1985; Proudfoot Trend in Biochem. Sci. 14: 105-110, 1989; and Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; which are incorporated herein by reference). The isolated DNA molecules of the present invention are useful in producing recombinant human GFAT. Thus, the present invention provides the advantage that human GFAT is produced in high quantities that may be readily purified for use in the disclosed methods for detecting compounds capable of inhibiting GFAT activity. GFAT, the first enzyme in the hexosamine pathway, catalyzes the formation of glucosamine-6-phosphate from fructose-6-phosphate and glutamine.

DNA molecules encoding GFAT may be isolated using standard cloning methods such as those described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, (1982); which is incorporated herein by reference), Sambrook et al. (Molecular Cloning:

A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, (1989); which is incorporated herein by reference) or Mullis et al. (U.S. Patent No. 4,683,195; incorporated herein by reference). Alternatively, GFAT coding sequences may be synthesized using standard techniques that are well known in the art, such as by synthesis on a DNA synthesizer.

Sequence ID NO: 1 and Sequence ID NO: 2 disclose a representative nucleotide sequence and deduced amino acid sequence of human GFAT. Analysis of the sequence discloses a primary translation product of 681 amino acids. As will be recognized by those skilled in the art, minor variations in the amino acid sequence of GFAT may occur. Such variations may be due to, for example, genetic polymorphisms or minor proteolysis. Sequence variations may also be introduced by genetic engineering techniques.

DNA molecules encoding GFAT or portions thereof may be used, for example, to directly detect GFAT sequences in cells. Such DNA molecules are generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences and will generally comprise from about 14 nucleotides to about 25 or more nucleotides, sometimes 40 to 60 nucleotides, and in some instances a substantial portion or even the entire cDNA of a GFAT gene. The synthetic oligonucleotides of the present invention are at least 85% homologous to a corresponding DNA sequence of the human glutamine:fructose-6-phosphate amidotransferase of Sequence ID NO: 1 or a complementary sequence thereto. For use as probes, the molecules are labeled to provide a detectable signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle, etc. according to methods known in the art. DNA molecules used within the present invention may be labeled and used in a hybridization procedure similar to the Southern or dot blot. As will be understood by those

skilled in the art, conditions that allow the DNA molecules of the present invention to hybridize to GFAT sequences or GFAT-like sequences may be determined by methods well known in the art and reviewed, for example, by Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; which is incorporated herein by reference). Those skilled in the art will be capable of varying hybridization conditions (i.e. stringency of hybridization) of the DNA molecules as appropriate for use in the various procedures by methods well known in the literature (see, for example, Sambrook et al., ibid., pages 11.45-11.53). The higher the stringency of hybridization, the lower the number of mismatched sequences are detected. Alternatively, lower stringency will allow related sequences to be identified.

Alternatively, human GFAT sequence variants may be identified using DNA molecules of the present invention and, for example, the polymerase chain reaction (PCR) (disclosed by Saiki et al., Science 239: 487, 1987; Mullis et al., U.S. Patent 4,686,195; Mullis et al., U.S. Patent 4,683,202, Orita et al., Proc. Nat'l Acad. Sci. USA 86: 2766-2770, 1989, Spinardi et al., Nucleic Acids Res. 19: 4009, 1991; which are incorporated by reference herein in their entirety) to amplify DNA sequences, which are subsequently detected by their characteristic size, such as on agarose gels, which may be sequenced to detect sequence abnormalities or which may be used within methods for detecting single strand conformation polymorphisms.

GFAT coding sequences are inserted into suitable expression vectors which are in turn introduced into prokaryotic and/or eukaryotic host cells. Expression vectors for use in carrying out the present invention comprise a promoter capable of directing the transcription of a cloned DNA and a transcriptional terminator. In some circumstances, it may be preferable

to direct GFAT into the secretory pathway of the host cell. In such a case, the expression vector would further comprise a secretory signal sequence capable of directing the secretion of the protein encoded by the cloned DNA downstream of the promoter and operably linked to the GFAT coding sequence.

The present invention also encompasses antisense oligonucleotides and "antisense" expression vectors capable of directing the transcription of antisense mRNA, which is complementary to mRNA encoding GFAT protein, capable of hybridizing to part or all of the endogenous GFAT-encoding mRNA. These antisense expression vectors thus transcribe sequences that are capable of preventing the translation of GFAT mRNA in a host cell thus reducing GFAT expression levels. It may be advantageous to utilize antisense sequences as described herein in pancreatic β-cells to reduce GFAT expression levels. Preferable sequences for use in antisense vectors are those sequences which inhibit the translation of GFAT mRNA in host cells that have been transfected or transformed with the antisense vector and include the 5' non-coding region of GFAT and the sequences that hybridize to the translation start AUG. Thus the antisense mRNA, which corresponds to a GFAT DNA sequence, may contain less than the entire length of GFAT sequence and may contain nucleic acid changes that do not inhibit hybridization to GFAT mRNA but significantly reduce the translation of the mRNA into GFAT. Antisense GFAT oligonucleotide sequences are preferably obtained from the 5' non-coding region and are preferably between 10 and 25 nucleotides in length, most preferably 18 nucleotides in length. Antisense GFAT expression vectors may be prepared by inserting a GFAT sequence in the opposite orientation relative to the transcriptional promoter in the expression vectors discussed in detail herein. The selection of suitable promoters, terminators, and vector sequences are within the level of

ordinary skill in the art (for review see Mirabelli et al., Anti-Cancer Drug Des. 6: 647-661, 1991; Crooke, Anti-Cancer Drug Des. 6: 609-646, 1991; James, Antiviral Chem. Chemother. 2: 191-214, 1991).

Host cells for use in practicing the present invention include prokaryotic and eukaryotic cells. Preferred prokaryotic host cells for use in carrying out the present invention are strains of the bacteria E. coli, although Bacillus and other genera are also useful. Eukaryotic host cells for use in the present invention include mammalian, avian, plant, insect, and fungal cells. Fungal cells, including species of yeast (e.g., Saccharomyces spp., Schizosaccharomyces spp.) or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells within the present invention. Strains of the yeast Saccharomyces cerevisiae are particularly preferred.

For bacterial host cells, plasmids suitable for transforming bacteria include pBR322 (Bolivar et al., Gene 2: 95-113, 1977), the pUC plasmids (Messing, Meth. Enzymol. 101:20-77, 1983), Vieira and Messing, Gene 19: 259-268, 1982), pCQV2 (Queen, J. Mol. Appl. Genet. 2: 1-10, 1983), pIC vectors (Marsh et al., Gene 32: 481-485, 1984), and derivatives thereof. Suitable vectors may be purchased from commercial suppliers (i.e., from GIBCO-BRL (Gaithersburg, MD), Boehringer Mannheim (Indianapolis, IN), and New England Biolabs (Beverly, MA)).

Appropriate promoters include the trp (Nichols and Yanofsky, Meth. Enzymol. 101: 155-164, 1983), lac (Casabadan et al., J. Bacteriol. 143: 971-980, 1980), and phage λ promoter systems (Queen, ibid.). A particularly preferred promoter is the tac promoter (Amann et al., Gene 40: 183, 1985 and de Boer et al., Proc. Natl. Acad. Sci. USA 80: 12, 1983). Bacterial expression vectors will also include a ribosome-binding site upstream of the initiation codon. Ribosome-binding sites, also known as Shine-Delgarno sequences (Shine and Delgarno, Nature:

254: 34, 1975 and Steitz, in Biological regulation and development: Gene expression, R.F. Goldberger, ed., Vol 1, p. 349, Plenum Publishing, NY, 1979), are complementary to the 3' terminus of the E. coli 16S RNA and may be inserted by in vitro mutagenesis, ligation of a linker sequence or PCR-induced mutagenesis.

The choice of a suitable bacterial host cell is well within the level of ordinary skill in the art. Techniques for transforming bacterial host cells and expressing foreign genes cloned in them are well known in the art (see e.g., Maniatis et al., eds. , Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982; and Sambrook et al., ibid.). Methods for the recovery of the proteins in biologically active forms from bacteria are discussed in U.S. Patents Numbers 4,966,963 and 4,999,422, which are incorporated herein by reference.

For yeast host cells, suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76: 1035-1039, 1978), YEp13 (Broach et al., Gene 8: 121-133, 1979), POT vectors (Kawasaki et al, U.S. Patent No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibits a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al., ibid.), URA3 (Botstein et al., Gene 8: 17, 1970), HIS3 (Struhl et al., ibid.) or POT1 (Kawasaki et al., ibid.). Another suitable selectable marker is the chloramphenicol acetyl transferase (CAT) gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al.,

J. Biol. Chem. 255: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983). In this regard, particularly preferred promoters are the TPI1 promoter (Kawasaki, U.S. Patent No. 4,599,311) and the ADH2-4^{c} promoter (Russell et al., Nature 304: 652-654, 1983; Irani and Kilgore, U.S. Patent Application Serial No. 07/784,653, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi Aspergillus (McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from Aspergillus nidulans glycolytic genes, such as the ADH3 promoter (McKnight et al., EMBO J. 4: 2093-2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., ibid., 1985). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (Proc. Natl. Acad. Sci. USA 75: 1929-1933, 1978), Yelton et al. (Proc. Natl. Acad. Sci. USA 81: 1740-1747, 1984), and Russell (Nature 301: 167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and

selectable marker is well within the level of ordinary skill in the art.

To optimize production of recombinant human GFAT in host cells and to facilitate purification of the protein, it may be preferable to use host cells that are deficient in the native host GFAT analog. In E. coli, for example, it may be preferable to use a host cell containing a genetic defect in the glmS gene, the glutamine synthetase gene. E. coli strains carrying glmS$^-$ mutations have been described by Wu and Wu (J. Bact. 105: 455-466, 1971) and Dutka-Malen et al. (Biochimie 70: 287-290, 1988) among others. Yeast strains defective in glutamine:fructose-6-phosphate amidotransferase activity, such as gcn1 mutants may be obtained, for example, from the Yeast Genetic Stock Center (Department of Molecular and Cellular Biology, Division of Genetics, University of California at Berkeley). It may be preferable to disrupt the E. coli glmS gene or the Saccharomyces cerevisiae gcn1 gene using methods well established in the literature (see Rothstein, Methods in Enzymology 101: 202-211, 1981; which is incorporated herein by reference). The DNA sequence encoding the glnS gene has been disclosed by, for example, Walker et al. (Biochem. J. 224: 799-815, 1984; which is incorporated herein by reference), and the DNA sequence encoding the S. cerevisiae glutamine:fructose-6-phosphate amidotransferase gene has been disclosed, for example, by Watzele and Tanner (J. Biol. Chem. 264: 8753-8758, 1989; which is incorporated herein by reference). It is well within the level of ordinary skill to clone the E. coli glmS or the S. cerevisiae GCN1 gene using methods such as those essentially described in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2 ed., Cold Spring Harbor, New York, 1989) and use such DNA segments within methods for disrupting the native host gene as generally described above.

To optimize production of the heterologous proteins in yeast, it is preferred that the host strain carries a mutation, such as the yeast pep4 mutation (Jones, Genetics 85: 23-33, 1977), which results in reduced proteolytic activity. To optimize secretion of the heterologous proteins from yeast cells, it is preferred that the host strain carries a mutation in the SSC1 gene (Smith et al., U.S. Patent 5,057,416; which is now referred to as PMR1; Rudolph et al., Cell 58: 133-146, 1989) which results in the increase in secretion of heterologous proteins. It may be advantageous to disrupt the PMR1 gene. For secretion of foreign genes from yeast host cells, it may also be preferable to utilize a host cell that contains a genetic deficiency in at least one gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, such a genetic deficiency will be in either the MNN9 gene or the MNN1 gene or both (described in pending, commonly assigned U.S. Patent Application Serial No. 07/189,547, which is incorporated by reference herein in its entirety). Most preferably, the yeast host cell contains a disruption of both the MNN1 and MNN9 genes. A yeast strain containing disruptions of both the MNN1 and MNN9 strains was deposited with the American Type Culture Collection (Rockville, MD) under Accession number 20996. Yeast host cells having such defects may be prepared using standard techniques of mutation and selection. Ballou et al. (J. Biol. Chem. 255: 5986-5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized yeast cells were screened using fluoresceinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cultured mammalian cells for use in the present invention include the 3T3-L1 (ATCC CCL 92.1), COS-1 (ATCC CRL 1650), BHK, and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 6: 59-72, 1977) cell lines. A preferred BHK cell line is the BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314). In addition, a number of other mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CCL 29.1), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci USA 77: 4216-4220, 1980).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41: 521-530, 1985) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1: 854-864, 1981). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_K promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81: 7041-7045, 1983; Grant et al., Nuc. Acids Res. 15: 5496, 1987) and a mouse V_H promoter (Loh et al., Cell 33: 85-93, 1983). A particularly preferred promoter is the major late promoter from Adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2: 1304-13199, 1982). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or

immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9: 3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, Cell 33: 717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7: 603, 1981; Graham and Van der Eb, Virology 52: 456, 1973; which are incorporated by reference herein in their entirety). Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., EMBO J. 1: 841-845, 1982) and cationic lipid transfection using commercially available reagents including the Boehringer Mannheim Transfection-Reagent (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl ammonium-methylsulfate; Boehringer Mannheim, Indianapolis, IN) or LIPOFECTIN reagent (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride and dioleoyl phosphatidylethanolamine; GIBCO-BRL, Gaithersburg, MD) using the manufacturer-supplied directions, may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer

resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Promoters, terminators and methods suitable for introducing expression vectors encoding GFAT into plant, avian and insect cells are well known in the art. The use of baculoviruses, for example, as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (Pestic. Sci. 28: 215-224, 1990). The use of Agrobacterium rhizogenes as vectors for

expressing genes in plant cells has been reviewed by Sinkar et al. (J. Biosci. (Bangalore) 11: 47-58, (1987)).

To direct proteins of the present invention into the secretory pathway of the host cell, at least one signal sequence is operably linked to the DNA sequence of interest. The choice of suitable signal sequences for a particular host cell is within the level of ordinary skill in the art. Preferred signals for use in E. coli include the E. coli phoA signal sequence (Oka et al., Proc. Natl. Acad. Sci. USA 82: 7212, 1985). In fungal cells preferred signal sequences include the alpha factor signal sequence (pre-pro sequence; Kurjan and Herskowitz, Cell 30: 933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake, EP 116,201), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670; Welch et al., U.S. Patent No. 5,037,743), the SUC2 signal sequence (Carlson et al., Mol. Cell. Biol. 3: 439-447, 1983). In cultured mammalian cells preferred signal sequences include the α-1-antitrypsin signal sequence (Kurachi et al., Proc. Natl. Acad. Sci. USA 78: 6826-6830, 1981), the α-2 plasmin inhibitor signal sequence (Tone et al., J. Biochem. (Tokyo) 102: 1033-1042, 1987) and the tissue plasminogen activator leader sequence (Pennica et al., Nature 301: 214-221, 1983). Alternatively, a secretory signal sequence may be synthesized according to the rules established, for example, by von Heinje (Eur. J. Biochem. 133: 17-21, 1983; J. Mol. Biol. 184: 99-105, 1985; Nuc. Acids Res. 14: 4683-4690, 1986).

Signal sequences may be used singly or may be combined. For example, a first signal sequence may be used singly or in combination with a sequence encoding the third domain of Barrier (described in U.S. Patent 5,037,743, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence

of interest or 5' to the DNA sequence and in proper reading frame with both the signal sequence and the DNA sequence of interest.

Host cells containing DNA constructs of the present invention are then cultured to produce GFAT. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the particular host cell employed. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker present on the DNA construct or co-transfected with the DNA construct.

Bacterial cells, for example, are preferably cultured in a complex, chemically undefined, medium comprising a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and in appropriate cases an antibiotic for the selection of plasmid-containing cells. Bacterial cells transformed with expression units driven by inducible promoters are preferably cultured in a chemically defined medium that is either supplemented with the inducing substance or is lacking a nutrient whose deficiency induces the promoter. For example, bacterial cells transformed with an expression unit driven by the inducible tac promoter are preferably cultured in complex medium with the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of the desired protein from the promoter.

Yeast cells are preferably cultured in a chemically defined medium, comprising a carbon source, a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 6.5. Methods for

maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M, preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The GFAT produced according to the present invention may be purified by affinity chromatography on an antibody column using antibodies directed against GFAT. Antibodies generated against GFAT may also be useful in detecting the presence of GFAT in cell lysates in immunological assays (reviewed by, for example, Sambrook et al., ibid.; which is incorporated by reference herein in its entirety) such as enzyme-linked immunosorbant assays and Western blot assays (Towbin et al. Proc. Natl. Acad. Sci. USA 76: 4350, 1979). The antibodies prepared against GFAT are capable of specifically binding to GFAT by which is meant that the antibodies against GFAT react with epitopes that are specific for GFAT. Antibodies directed against GFAT or portions of GFAT may be generated using conventional techniques. Methods for fusing lymphocytes and immortalized cells and generating monoclonal antibodies from the resultant hybridomas are disclosed by Kohler and Milstein (Nature 256: 495-497, 1975; Eur. J. Immunol. 6: 511-519, 1976) and reviewed by, for example, Hurrell (Monoclonal Hybridoma Antibodies: Techniques and Applications CRC Press, Inc., 1982). Portions of GFAT for use as immunogens may be chemically synthesized, such

as by the solid-phase method of Barany and Merrifield (in The Peptides Vol. 2A, Gross and Meienhofer, eds, Academic Press, NY, pp. 1-284, 1979) or by use of an automated peptide synthesizer.

Additional purification may be achieved by conventional chemical purification means, such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, NY (1982), which is incorporated herein by reference) and may be applied to the purification of the recombinant GFAT described herein. Substantially pure recombinant GFAT of at least about 50% is preferred, at least about 70-80% more preferred, and 95-99% or more homogeneity most preferred. The present invention provides for the production of human GFAT essentially free of other proteins of human origin.

It will be understood by those skilled in the art that GFAT or portions thereof may be synthesized following any suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution addition. Synthetic GFAT or portions thereof of the present invention may be prepared by hand synthesis or using a suitable peptide synthesizer such as an Applied Biosystems (Foster City, CA) Model 431A peptide synthesizer or the like.

Within the present invention, recombinant human GFAT is used in assays to detect compounds capable of inhibiting GFAT activity. These assays will generally include the steps of (a) exposing a test substance to human GFAT in the presence of fructose-6-phosphate and glutamine under physiological conditions and for a time sufficient to allow the test substance to inhibit glutamine:fructose-6-phosphate amidotransferase activity; and (b) detecting a reduction in activity of GFAT and

therefrom determining the presence in the test substance of a compound which inhibits human GFAT.

Conditions and times sufficient for the inhibition of GFAT activity will vary with the particular assay used; however, conditions suitable for inhibition will generally be between 4°C and 55°C, preferably between 30°C and 40°C, under physiological conditions. As used herein, "physiological conditions" indicates conditions approximating the normal environment of cell-associated GFAT, and includes cell culture media and buffered, low-salt solutions within a pH range of between 5 and 9, preferably between 6.8 and 8.0. Sufficient time for the inhibition and response will be between 5 and 60 minutes after exposure, with 15-30 minutes being particularly preferred. However, sufficient time will also be dependent on parameters of the assay such as protein concentrations and substrate concentrations. Sufficient time for the inhibition of GFAT activity may be determined by varying a particular parameter such as substrate concentration in the assay and stopping the assay at specific time points. A plot of the varied parameter versus the assay time in assays containing no inhibitors may then be prepared and a time sufficient for inhibition may be chosen from the linear portion of the graph.

Recombinant GFAT for use within the assays of the present invention may be purified according to methods well known in the literature, may be partially purified or may be utilized within crude cell extracts. It may be preferable to purify the recombinant GFAT for use within the disclosed assays. Purification steps include ion exchange chromatography on Q fast flow, hydrophobic chromatography on organomercurial agarose and gel filtration. Purification of GFAT has been described, for example, by Dutka-Malen et al. (Biochimie 70: 287-290, 1988) and Badet et al. (Biochemistry 26: 1940-1948, 1987).

Glutamine:fructose-6-phosphate amidotransferase catalyzes the formation of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. Glutamate is a by-product of the GFAT-catalyzed reaction. Assays for measuring GFAT activity generally rely on the measurement of the production of glucosamine-6-phosphate or the production of glutamate. Glucosamine-6-phosphate production may be measured by the assay essentially described by Ghosh et al. (J. Biol. Chem. 255: 1265, 1960; which is incorporated by reference herein in its entirety) and Zalkin et al. (Meth. Enzymol. 113: 278-281, 1985; which is incorporated by reference herein in its entirety), which relies on detecting the color development resulting from the reaction of glucosamine-6-phosphate with ρ-dimethylaminobenzaldehyde. The production of the byproduct glutamate from the GFAT-catalyzed reaction may be measured using methods essentially described by Shijo et al. (J. Biochem. (Tokyo) 66: 175, 1969), Traxinger et al. (J. Biol. Chem. 266: 10148-10154, 1991) or Lund (Methods of Enzymatic Analysis, Vol. III, Bergmeyer, ed., 357-363, 1985). Such assays rely on the reduction of adenine dinucleotides in the presence of glutamate and glutamate dehydrogenase. Callahan et al. (Anal. Biochem. 115: 347-352, 1981; which is incorporated by reference herein in its entirety) disclose a radioisotope assay for GFAT that relies on measuring the synthesis of radiolabeled glucosamine-6-phosphate.

In an exemplary assay, between 1 ng and 1 μg of human GFAT, preferably approximately between 0.5 and 1 μg of human GFAT, is inoculated into each well of a 96-well microtiter plate. Ethyl acetate-extracted test substances are diluted to between approximately 0.1X and 0.5X, and the diluted test substances are added to each well. Reaction mixtures are prepared such that each well contains between 10 μM and 20 mM fructose-6-phosphate, preferably 10 mM fructose-6-phosphate; 0.25 μCi ^{3}H-

fructose-6-phosphate; 10 mM L-glutamine; 30 mM sodium phosphate buffer (pH 7.5); 1 mM EDTA and 1 mM DTT. The plate is vortexed and incubated at 37°C for a time sufficient to allow ^{3}H-glucosamine-6-phosphate production, preferably between 10 minutes and 1 hour. After incubation, each reaction is stopped by the addition of an equal volume of 1.0 M sodium borate (pH 8.5).

The ^{3}H-glucosamine-6-phosphate content of each sample is assayed by cross-linking the ^{3}H-glucosamine-6-phosphate to a solid phase support that can be removed by filtration and counted by scintillation. One such support is a nylon powder (25,000, 3/32 inch diameter polished or unpolished nylon beads; The Hoover Group, Sault St. Marie, MI) that has been treated with hexane diamine (Aldrich Chemical) in the presence of triethyloxonium tetrafluoroborate (Aldrich Chemical) essentially as described by Van Ness et al. (Nuc. Acids Res. 10: 3345-3349, 1991). Briefly, nylon beads (25,000, 3/32 inch diameter polished or unpolished nylon beads) in anhydrous 1-methyl-2-pyrrolidinone are stirred for five minutes at room temperature. Triethyloxonium tetrafluoroborate is added to the mixture, which is stirred for an additional thirty minutes at room temperature. After stirring, the liquid is decanted, and the beads are quickly washed four times with 1-methyl-2-pyrrolidinone. The beads are stirred for twelve to twenty-four hours in 80% 1-methyl-2-pyrrolidinone, 20% hexane diamine, after which the solution is decanted, and the beads are washed with 1-methyl-2-pyrrolidinone follwoed by copious amounts of water. The beads are dried in vacuo for four to five hours. The amino groups on the derivatized powder are activated by the addition of a 10-fold excess of cyanuric chloride (Fluka, Buchs, Suisse) prepared at 150 mg/ml in acetonitrile for thirty minutes at room temperature followed by multiple washes with 0.5 M borate buffer.

The derivatized nylon powder is added to the test reactions, and the primary amines present in the reaction mixture, including those on ^{3}H-glucosamine-6-phosphate quantitatively crosslink with the activated nylon powder. The bound ^{3}H-glucosamine-6-phosphate is recovered by filtration on glass fiber mats (Pharmacia), and the filters are counted on a Packard β-plate scintillation counter. A reduction in ^{3}H-glucosamine-6-phosphate production in the presence of a test sample relative to the ^{3}H-glucosamine-6-phosphate produced in the absence of the test substance indicates that the substance is a GFAT inhibitor.

In instances where recombinant GFAT preparations contain glucose-6-phosphate isomerase such as in cell extracts or in partially purified enzyme preparations, it will be necessary to completely remove the glucose-6-phosphate isomerase because it will rapidly deplete the ^{3}H-fructose-6-phosphate in the assay. In such cases, the recombinant GFAT may be purified away from the isomerase using a single-step *p*-hydroxymercuribenzoate affinity chromatography method (described by Hosoi et al. (Biochem. Biophys. Res. Comm. 85: 558-563, 1978; which is incorporated by reference herein in its entirety) wherein the recombinant GFAT is desorbed from the column matrix by elution with 20 mM DTT.

As will be evident to one skilled in the art, incubation periods may be shortened or lengthened to optimize the assay by the application of routine experimentation. In one embodiment of the invention, GFAT inhibitors are detected through their ability to reduce the conversion of radiolabeled glutamine to the byproduct radiolabeled glutamate. In another embodiment, GFAT inhibitors are detected through their ability to reduce the conversion of glutamine to glutamate as measured by the reduction of 3-acteylpyridine adenine dinucleotide by glutamate dehydrogenase essentially as

described by Traxinger and Marshall (J. Biol. Chem. 266: 10148-10154, 1991). In yet another embodiment of the invention, GFAT inhibitors are detected through their ability to reduce the conversion of ^{3}H-fructose-6-phosphate to ^{3}H-glucosamine-6-phosphate.

Inhibitors of GFAT activity are those substances that inhibit between 90% and 20% of GFAT activity levels, preferably between 80% and 50% of GFAT activity levels, relative to GFAT activity in the absence of the inhibitor. A preferred GFAT inhibitor is a substance that provides between 90% and 20% inhibition, preferably between 80% and 50% inhibition of GFAT activity and does not confer any adverse physiological side effects. Inhibitors of GFAT are administered at a level that results in a reduced level of GFAT activity and a concomitant increase in glucose utilization without any adverse physiological side effects. An amount adequate to accomplish this is defined as a "therapeutically effective dose."

Inhibitors of GFAT of the present invention are prepared in compositions for parenteral and/or oral administration (i.e., intravenously, subcutaneously, or intramuscularly). Compositions of GFAT inhibitors for parenteral administration generally comprise a solution of the inhibitor dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, 20-30% glycerol and the like. These compositions may be sterilized by conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting

agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of GFAT inhibitor in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLES

Example 1 -- Cloning of human GFAT cDNA sequences

Human pheochromocytoma specimens were obtained from multiple patients and pooled. The tissue samples were frozen in liquid nitrogen, fragmented by mortar and pestle in liquid nitrogen and solubilized in RNA extraction buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1% b-mercaptoethanol, 0.5% sodium lauryl sarcosinate). The fragmented tissue was homogenized for 20 seconds using a tissue homogenizer. Phenol:chloroform:isoamyl alcohol (50:48:2) was added, and the mixture was vortexed and centrifuged. The RNA was precipitated with isopropanol. The pellet was resuspended in RNA extraction buffer and precipitated again with isopropanol. The RNA pellet was sequentially washed with 75% and 100% ethanol. Poly(A)+ RNA was enriched using oligo d(T)-cellulose column chromatography as described by Sambrook et al., eds. (Molecular Cloning:

A Laboratory Manual, vol. 1, 7.26-7.29, Cold Spring Harbor Laboratory Press, 1989).

First strand cDNA was synthesized from the poly(A)+ RNA by first incubating 1.0 μg of the poly(A)+ RNA at 65°C for 3 minutes in 5 mM Tris-HCl (pH 7.0), 0.05 mM EDTA. The RNA was cooled on ice, and the cDNA synthesis reaction was primed with 5 pmol of oligonucleotide ZC2487 (Sequence ID NO: 3) in a 10 μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each deoxynucleotide triphosphate, and 200 units of MMLV (RNase H^-) reverse transcriptase (GIBCO-BRL; Gaithersburg, MD). The reaction mixture was incubated at 45°C for 1 hour. After the incubation the mixture was diluted with 180 μl of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA and stored at 4°C.

GFAT cDNA sequences were amplified from the first strand cDNA using degenerate oligonucleotide primers encoding GFAT DNA sequences by polymerase chain reaction (PCR). Three microliters of the cDNA solution and 1 μM each of oligonucleotide ZC3866 and ZC3868 (Sequence ID NO: 4 and Sequence ID NO: 6) were combined in a reaction volume of 50 μl containing 200 μM of each deoxynucleotide triphosphate and 1X Thermus aquaticus (Taq) buffer (Promega Corporation, Madison, WI). The reaction was heated to 95°C for 5 minutes. After cooling, 2.5 units of Taq DNA polymerase (Promega) was added, and the reaction mixture was overlaid with mineral oil. The PCR reaction was run for 40 cycles (thirty seconds at 95°C, thirty seconds at 42°C and sixty seconds at 72°C) followed by a 10 minute incubation at 72°C.

An aliquot of the amplification reaction was used for a second PCR reaction using different oligonucleotide primers, each of which contained a 5' tail of 10 nucleotides encoding convenient restriction enzyme sites for subcloning. A one microliter aliquot of the first PCR reaction was combined with 1 μM each of the

oligonucleotide primers ZC3867 and ZC3869 (Sequence ID NO: 5 and Sequence ID NO: 7) in a final reaction volume of 50 µl that contained 200 µM of each deoxynucleotide triphosphate and 1X Taq buffer (Promega). The reaction mixture was heated to 95°C for 5 minutes, after which the mixture was allowed to cool, and 2.5 units of Taq polymerase were added. The mixture was then overlaid with mineral oil. The PCR reaction was run for 40 cycles (thirty seconds at 95°C, thirty seconds at 42°C and sixty seconds at 72°C) followed by a 10 minute incubation at 72°C.

A 0.3 kb PCR reaction product was isolated by agarose gel electrophoresis. The purified fragment was ligated into pCR1000 (Invitrogen, San Diego, CA) and electroporated into E. coli strain DH10B (GIBCO-BRL) using a Bio-Rad Electroporator (Bio-Rad Laboratories; Richmond, CA) at 400 ohms, 25 µfarads and 2000 volts. Aliquots of the transformed cells were plated onto LB plates containing 50 mg/l Kanamycin (Sigma Chemical Co.; St. Louis, MO).

Four colonies, designated 7-1 through 7-4, were selected and analyzed for inserts by using the oligonucleotide primers ZC3867 and ZC3869 (Sequence ID NO: 5 and Sequence ID NO: 7) in independent PCR reaction mixtures each of which included an inoculum from a clone as the source of template DNA. The PCR reaction was carried out as described above with the exception that only 30 cycles of amplification were carried out. Sequence analysis of the cDNA inserts revealed a 0.3 kb sequence with homology to the yeast and E. coli GFAT sequences.

Example 2 -- Isolation of a human GFAT cDNA clone from HepG2 cells

A cDNA coding for a portion of human GFAT was obtained from a λgt11 cDNA library prepared from HepG2

cells as described by Hagen et al. (Proc. Natl. Acad. Sci. USA 83: 2412-2416, 1986). The library was screened for sequences corresponding to the human GFAT DNA using clone 7-1 (described previously). Approximately one million phage plaques were screened, and one positive plaque was identified.

The phage plaques were fixed to nylon filters (ICN Biomedicals, Inc.; Irvine, CA) by placing 10 cm filters onto petri plates that had been plated with the library. The filters were removed and placed in lysing solution (0.5 M NaOH and 1.5 M NaCl). After five minutes, the filters were neutralized for five minutes in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl. After neutralization, the filters were rinsed in 2X SSPE (0.36 M NaCl, 20 mM NaH_2PO_4 (pH 7.4), and 2 mM EDTA (pH 7.4) for one minute. The filters were allowed to air dry and were then baked in a vacuum oven at 80°C for 2 hours. Following baking, the filters were prewashed in 5X SSC (43.8 g NaCl, 22 g sodium citrate dissolved in distilled water to a final volume of one liter and pH adjusted to 7.0). The filters were prehybridized overnight at 37°C in 50% formamide containing Ullrich's buffer (25 ml of 50X Denhardt's (Sambrook et al., ibid. and Maniatis, ibid.); 5 ml of 10 mg/ml salmon sperm DNA; 25 mg of adenosine triphosphate; 25 ml of 1 M sodium phosphate (pH 7.0); 25 ml of 0.1 M sodium pyrophosphate; 125 ml of 20X SSC; 65 ml of sterile H_2O).

The PCR-generated DNA from clone 7-1 was used to prepare a probe using the MULTIPRIME Labeling Kit (Amersham; Arlington Heights, IL) according to the manufacturer's specifications. The filters were prehybridized in 50% formamide/Ullrich's buffer (described above) at 37°C for approximately 18 hours, then hybridized for approximately 24 hours at 37°C in 50% formamide/Ullrich's buffer containing the radiolabeled probe.

After hybridization, the filters were washed twice for 20 minutes each in a solution of 2X SSC, 0.1% sodium lauryl sarcosinate (SDS) at room temperature. A third wash was carried out in a solution of 0.5X SSC, 0.1% SDS at 50°C for 40 minutes. The filters were air dried, then exposed to X-ray film.

A plug of agar corresponding to a positive area on the autoradiograph was picked from the master plate into 500 µl TM solution (10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$) and 20 µl chloroform to release the phage. This isolate was designated 6-1. The phage were plaque-purified by replating on E. coli strain Y1090 cells. The filters were prepared, processed and probed as described above using the radiolabeled 7-1 PCR probe. The filters were washed twice in a solution of 2X SSC, 0.1% SDS at room temperature for 30 minutes. The filters were washed at 55°C and 65°C consecutively for 30 minutes each in 0.5X SSC, 0.1% SDS. The filters were air dried and then exposed to X-ray film.

Positive plaques corresponding to positive areas on the autoradiographs were picked into 500 µl TM solution (10 mM Tris-HCl (pH 7.4); 10 mM $MgCl_2$) and 20 µl chloroform, and the released phage were replated on to a lawn of Y1090 cells. A plate lysate was prepared from each clone to obtain a phage titer.

DNA was extracted from two clones, designated 6-1-1 and 6-1-3, using a liquid phage preparation. Y1090 cells were grown in NZY media (Sambrook et al., ibid.) to an OD_{600} of 5.6 and then infected with 5 x 10^6 plaque forming units (pfu) of phage. The cultures were shaken at 37°C for 5.5 hours. Ten milliliters of chloroform was added to each culture, and the cultures were shaken for 15 minutes at 37°C. The cultures were centrifuged at 5,000 rpm for 10 minutes in a Sorvall GSA rotor (DuPont Co.; Wilmington, DE). The supernatants were collected, and 460 µg of RNase A (Sigma) and 460 µg of DNase I (Sigma) were added to each supernatant sample. After a

30 minute incubation at room temperature, 26.8 grams of NaCl and 46 grams of PEG 8000 (Sigma) were added to each sample, and the samples were incubated at 4°C overnight. After the incubation, the samples were centrifuged in a Sorvall GSA rotor (DuPont Co.) as described above, and the pellets were each resuspended in 5 ml of a CsCl solution prepared by adding 67 g of CsCl to 82 ml SM (Maniatis et al., ibid.). The CsCl mixtures were spun in an SW55 rotor (Beckman Instruments, Inc.; Palo Alto, CA) at 35,000 rpm at 20°C overnight. The DNA bands corresponding to the phage DNA for each clone were extracted and pooled. The pooled DNA for each clone was ethanol precipitated at -20°C.

The pooled DNA was digested with Eco RI, and a 1.1 kb fragment was gel purified. The 1.1 kb DNA fragment and Eco RI-linearized pUC19 were ligated. The ligation mixture was used to electroporate E. coli strain DH10B cells. The electroporated E. coli were plated on LB plates containing 50 mg/l ampicillin. The presence of insert in selected transformants was determined by PCR amplification using oligonucleotides ZC4306 and ZC4307 (Sequence ID NO: 8 and Sequence ID NO: 9). The selected transformants were inoculated into separate reaction mixtures each of which contained 200 μM of each deoxynucleotide triphosphate and 1X Taq buffer in a final volume of 50 μl. The reaction mixtures were heated to 95°C for 5 minutes. The mixtures were allowed to cool and 2.5 units of Taq polymerase were added, and the mixtures were overlaid with mineral oil. The PCR reactions were run for 30 cycles (thirty seconds at 95°C, thirty seconds at 50°C and sixty seconds at 72°C) followed by a 5 minute incubation at 72°C. The PCR products were subjected to agarose gel electrophoresis. Transformants containing plasmids with insert exhibited 1.1 kb PCR reaction products.

Plasmid DNA was prepared from a transformant containing a plasmid with 1.1 kb insert. Sequence

analysis confirmed the insert size of 1.1 kb; however, the sequence did not extend to the initiation ATG. The insert, designated 6-1, was isolated as an Eco RI fragment, radiolabeled using the Megaprime Labeling kit (Amersham), and used to screen the HepG2 library for a full length human GFAT cDNA clone. Approximately one million lambda phage infected into E. coli Y1090 cells were screened as described previously. Fifteen potential positive plaques were isolated, reinfected at 500 pfu/plate and rescreened using 50% formamide/Ullrich's buffer hybridization solution containing 10% dextran sulfate and the radiolabeled probe. One plaque, designated 13.2, was found to hybridize to the 6-1 probe upon rescreening. Plaque purification was carried out as previously described using Starks buffer (5X SSC, 25 mM sodium phosphate (pH 6.5), 1X Denhardt's, 0.3 mg/ml salmon sperm DNA, 50% formamide, 10% dextran sulfate) in place of the Ullrich's hybridization buffer. Phage DNA was isolated from each clone as described above. The GFAT insert from each clone was excised by Eco RI digestion and gel purified as an approximately 3.1 kb fragment. The insert DNA was sequenced and was found to encode GFAT. A λ clone containing a 3.1 kb GFAT sequence was designated 13.2.3.

Plasmid pBS(+) (Stratagene Cloning Systems; La Jolla, CA) was digested with Eco RI and treated with calf alkaline phosphatase to prevent recircularization. The phosphatased plasmid was gel purified and used in a ligation reaction with the Eco RI-digested GFAT insert from λ clone 13.2.3. The ligation mixture was electroporated into E. coli strain DH10B, and the presence of insert in selected transformants was determined by PCR as described previously. Plasmid DNA was prepared from a positive transformant, and the DNA was analyzed by digestion with Bam HI and Eco RI to confirm the presence of the correct insert. A plasmid containing the 13.2.3 insert in plasmid pBS(+) was

designated pBSGFAT #1. The DNA sequence and deduced amino acid sequence of the GFAT coding sequence present in pBSGFAT #1 is shown in Sequence ID NO: 1 and Sequence ID NO: 2. Plasmid pBSGFAT #1 was deposited on March 25, 1992 with the American Type Culture Collection (12301 Parklawn Dr., Rockville, MD) as an E. coli transformant under Accession No. 68946.

Example 3 - Isolation of human genomic GFAT clones

Human genomic GFAT clones were obtained from a human lung fibroblast library (Stratagene Cloning Systems, lambda FIX II #944201). Approximately one million phage were adsorbed and plated with E. coli strain LE392 (Stratagene Cloning Systems). Plaques were lifted onto 1.2 μm BIOTRANS Nylon membranes (ICN Biomedicals, Inc.). The membranes were soaked for five minutes on filter paper saturated with Denaturing Buffer (0.5 N NaOH, 0.6 N NaCl). After denaturation, the filters were subjected to two sequential five-minute incubations in Neutralization Buffer (1.0 M Tris (pH 7.0), 1.5 M NaCl). After neutralization, the membranes were rinsed in a solution of 2x SSC, 0.1% SDS for ten minutes. After the rinse, the filters were blotted dry and baked for two hours at 80°C in vacuo. The membranes were prewashed at 42°C for one hour in 500 ml of 50 mM Tris (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.1% SDS. After prewashing, the membranes were prehybridized in approximately 60 ml of Starks buffer containing 0.1% SDS for three to four hours at 42°C. A radiolabelled probe was prepared by first digesting plasmid pBSGFAT with Eco RI and Pvu I. The digested DNA was subjected to agarose gel electrophoresis, and the 3.1 kb GFAT cDNA fragment was purified using GENE CLEAN (Bio 101; La Jolla, CA). After purification, the Eco RI fragment was radiolabelled using the MEGAPRIME Kit (Amersham). The prehybridized membranes were hybridized in approximately 60 ml of

Hybridization solution (Starks buffer containing 10% Dextran Sulfate, 0.1% SDS and approximately 4 X 10^7 CPM of the GFAT probe) overnignt at 42°C. After hybridization, the membranes were subjected to a first wash in 2x SSC, 0.1% SDS for twenty minutes at room temperature followed by two sequential twenty-minute washes at 55°C in 0.1x SSC, 0.1% SDS. After the final wash, the membranes were autoradiographed. Six potential positive plaques were identified.

Plugs of plaques encompassing the six potential positives were picked and adsorbed and plated with E. coli strain LE392 (Stratagene Cloning Systems). After incubation, the resultant plaques were screened as described above. The secondary screens revealed two positive plaques designated 1a and 15a. The two plaques, 1a and 15a, were then each plaque purified in tertiary screens using the 3.1 kb cDNA probe and conditions described above. Double-stranded DNA was isolated from each purified clone using the method developed by Grossberger (Nucleic Acids Res. 15: 6737, 1987; which is incorporated herein in its entirety). Briefly, plaques were picked into tubes containing 300 µl of adsorption buffer (10 mM $MgCl_2$,10 mM $CaCl_2$), 200 µl of an exponential culture of E. coli strain LE392, and L broth containing 0.4% maltose. The cultures were grown for 10 minutes at 37°C, after which 10 ml of L broth containing 10 mM $MgCl_2$ and 0.1% glucose was added to each tube. The tubes were shaken overnight at a 45° incline with the caps in the half-open position. After the overnight incubation, the tubes were centrifuged at 2000 rpm for 10 minutes. The supernatants were decanted and centrifuged in a SW41 rotor (Beckman) at 30,000 rpm for 30 minutes. The supernatants were discarded, and the phage pellets were suspended in 200 µl of SM (Maniatis et al., eds., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1982). The phage suspensions were each transferred to a microfuge tube and 200 µl of a freshly

made 1 mg/ml proteinase K (Boehringer Mannheim) solution was added to each tube. The tubes were then incubated for 2 hours at 37°C. The suspensions were extracted once with phenol and once with chloroform. The DNA was precipitated from the aqueous layer with 100 µl of 7.5 M ammonium acetate and 1 ml of 100% ethanol. The precipitates were pelleted by centrifugation, washed with ethanol and dried. The dried pellets were each resuspended in 100 µl of TE. The DNA from clone 1a produced an approximately 14 kb insert upon digestion with Sal I. The 14 kb fragment was gel purified and cloned into the Sal I site of pBS(+) (Stratagene). Two transformant clones containing the 14 kb insert in the pBS vector were designated pBSGFAT-1a(#2) and pBSGFAT-1a(#3). The DNA from clone 15a was digested with Sal I and found to contain an approximately 20 kb insert. Clone 15 a was digested with Xba I to produce two unique fragments of approximately 15 kb and 5 kb in size. The 15 kb and 5 kb Xba I fragments were each gel purified and subcloned into Xba I-linearized pBS(+) (Stratagene Cloning Systems). Transformants containing the 15 kb inserts in the pBS(+) vector were designated pBSGFAT-15aX1(#17) pBSGFAT-15aX1(#18). Transformants containing the 5 kb insert in the pBS(+) vector were designated pBSGFAT-15aX2(#25) and pBSGFAT-15aX2(#26). Restriction analysis of plasmids pBSGFAT-15aX1(#17), pBSGFAT-15aX1(#18), pBSGFAT-15aX2(#25), pBSGFAT-15aX2(#26), pBSGFAT-1a(#2) and pBSGFAT-1a(#3) suggested an overlap between clones 1a and 15a. All of the cDNA sequence lies within the subclone pBSGFAT-1a#2.

Example 4 - Expression of GFAT

The 2.0 kb fragment comprising the GFAT coding sequence was generated by PCR amplification using the 3.1 kb Eco RI fragment from λ clone 13.2.3 as a template. Oligonucleotide ZC4839 (Sequence ID NO: 12) was designed to encode the first 19 base pairs of the GFAT coding

sequence and included a 5' terminal Eco RI site. Oligonucleotide ZC4866 (Sequence ID NO: 13) was designed to anneal to the 19 3' terminal base pairs of the GFAT coding sequence and contained a second stop codon downstream of the coding sequence and a terminal Eco RI site on the 3' end of the primer. A 100 µl PCR reaction mixture was prepared containing 100 pmoles of each primer, 50 ng of the λ clone 13.2.3 template, 1x Taq Buffer (Promega), 200 µM dNTP's, and 5 units of Taq polymerase (Promega). The reaction mixture was amplified for 20 cycles (thirty seconds at 94°C, sixty seconds at 45°C and three minutes at 72°C). An aliquot of the PCR reaction was electrophoresed on an agarose gel, and a 2.0 kb fragment was isolated with GENE CLEAN (Bio 101) according to the manufacturer's directions. The 2.0 kb fragment was digested with Eco RI and was ethanol was precipitated for 10 minutes on ice. The digested DNA was pelleted by centrifugation and was resuspended in distilled water. Plasmid pPROK-1 (Clontech Laboratories, Inc.; Palo Alto, CA) was digested with Eco RI, treated with calf alkaline phosphatase to prevent recircularization, and gel purified with GENE CLEAN (Bio 101). The 2.0 kb Eco RI fragment and the Eco RI-linearized pPROK-1 were ligated, ethanol precipitated, and the precipitated DNA was transfected into E. coli strain DH10B by electroporation.

The presence of insert in selected transformants was determined by PCR amplification as described above using oligonucleotides ZC4804 and ZC4307 (Sequence ID NO: 11 and Sequence ID NO: 9), which were designed to hybridize to internal GFAT sequences. Plasmid DNA was prepared from transformants containing insert, and the DNA was digested with Eco RI and Sma I to determine which plasmids contained the insert in the proper orientation. A plasmid containing the GFAT coding sequence in the proper orientation relative to the tac

promoter in the vector pPROK-1 was digested with Eco RI to isolate the 2.0 kb fragment encoding human GFAT.

The 2.0 kb GFAT fragment was inserted into the vector ZMB-3 that had been linearized by digestion with Eco RI. Plamsid ZMB-3 is a derivative of the expression vector Zem228R. Plasmid Zem228 is a pUC18-based expression vector containing a unique Bam HI site for insertion of cloned DNA between the mouse metallothionein-1 (MT-1) promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, mouse neomycin resistance gene, and SV40 terminator. Zem228 was modified to delete the two Eco RI sites by partial digestion with Eco RI, blunting with DNA polymerase I (Klenow fragment) and dNTPs, and re-ligation. Digestion of the resulting plasmid with Bam HI followed by ligation of the linearized plasmid with Bam HI-Eco RI adapters resulted in a unique Eco RI cloning site. The resultant plasmid was designated Zem228R. Plasmid ZMB-3 is similar to Zem228R but contains the adenovirus 2 major late promoter, adenovirus 2 tripartite leader, and 5' and 3' splice sites substituted for the MT-1 promoter. The ligation mixture was electroporated into E. coli strain DH10B cells, and selected transformants were screened for the presence the insert as described above. A plasmid containing the 2.0 kb insert was obtained and designated pZMBGFAT.

The 5' portion of the GFAT cDNA was altered to insert an Eco RI site immediately upstream of the translation initiation codon. Plasmid pBSGFAT was used as a template for PCR reaction which generated an approximately 0.7 kb fragment encoding the 5' portion of the GFAT cDNA. Plasmid pBSGFAT was digested with Eco RI to obtain the approximately 3.1 kb GFAT cDNA. Oligonucleotide ZC6089 (Sequence ID NO: 18) was designed to encode a 5' Eco RI restriction site immediately preceding the first 16 nucleotides of the GFAT coding sequence. Oligonucleotide ZC6090 (Sequence ID NO: 19)

was designed to anneal to GFAT coding sequence from nucleotide 801 to nucleotide 823 of Sequence ID NO: 1. A 100 µl PCR reaction mixture was prepared containing 100 pmoles of each primer, 50 ng of the 3.1 kb Eco RI fragment from pBSGFAT, 1x Taq Buffer (Perkin Elmer Cetus), 200 µM dNTP's, and 5 units of Taq polymerase (Perkin Elmer Cetus). The reaction mixture was amplified for five cycles (thirty seconds at 94°C, sixty seconds at 45°C and sixty seconds at 72°C) followed by 20 cycles (thirty seconds at 94°C, sixty seconds at 58°C and sixty seconds at 72°C). An aliquot of the PCR reaction was electrophoresed on an agarose gel, and an approximately 0.7 kb fragment was isolated with GENE CLEAN (Bio 101) according to the manufacturer's directions. The gel-purified PCR fragment was digested with Eco RI and Sma I to isolate the approximately 0.6 kb fragment. The 3' GFAT coding sequence was obtained as a 1.4 kb Sma I-Eco RI fragment from plasmid pZMBGFAT. The Eco RI-Sma I PCR fragment and the Sma I-Eco RI fragment from plasmid pZMBGFAT were ligated into Eco RI linearized pPROK-1 (Clontech). The ligation mixture was electroporated into E. coli strain DH10B cells, and selected transformants were screened for the presence the insert as described above. Plasmid containing the 2.0 kb insert were obtained and were designated pPROKGFAT #18 and pPROKGFAT #25. The sequence of the cDNA in each plasmid was confirmed by sequence analysis.

The cDNA insert present in plasmid pPROKGFAT #18 was inserted into plasmid pZMB3 to obtain a mammalian expression vector. Plasmid pPROKGFAT #18 was digested with Eco RI, and the approximately 2.0 kb fragment containing the GFAT coding sequence was isolated by gel purification. The 2.0 kb Eco RI fragment was ligated with pZMB3 that had been linearized by digestion with Eco RI and treated with calf alkaline phosphatase to prevent recircularization. The ligation mixture was precipitated in the presence of approximately 20 µg of glycogen. The

precipitate was resuspended in water, and electroporated into E. coli strain DH10B cells (10 kV, 200 ohms, 25 μF). Transformants were selected in the presence of ampicillin. Clones containing the GFAT cDNA insert in the correct orientation relative to the promoter were identified by PCR amplification of portions of colonies. Portions of colonies of selected transformants were picked into 50 μl reaction mixtures containing 0.2 mM of each deoxyribonucleic acid, 1x PCR buffer (Perkin Elmer Cetus), 2 μM each of oligonucleotides ZC2435 and ZC5192 (Sequence ID NO: 16 and Sequence ID NO: 17, respectively) and 2.5 units of Taq polymerase (Perkin Elmer Cetus). Oligonucleotide ZC2345 (Sequence ID NO: 16) is a sense primer corresponding to sequences in the Adenovirus major late promoter present in pZMB3, and oligonucleotide ZC5192 (Sequence ID NO: 17) is an antisense primer corresponding to sequences in the GFAT cDNA. The PCR reactions were run for 30 cycles (forty-five seconds at 94°C, forty-five seconds at 58°C and forty-five seconds at 72°C) followed by a 5 minute incubation at 72°C. Aliquots of 10 μl were analyzed on a 1.5% agarose gel for the presence of an approximately 440 bp band indicating a correctly oriented cDNA insert. Plasmids pZMGFAT2-2 and pZMGFAT2-5 were identified as having the GFAT cDNA insert in the correct orientation relative to the promoter in pZMB3.

Plasmids pZMGFAT2-2 and pZMGFAT2-5 are transfected into suitable cultured mammalian cells using the Promega Transfectam Reagent (Promega Corp.; Madison, WI) according to the manufacturer-supplied directions. Stable clones of transfectants are selected in media containing G418. GFAT activity is assayed in selected transfectants.

Example 5 - Activity Assays

Fifty microliters of an overnight culture of XL1-BLUE transformant containing pPROKGFAT #18 is

inoculated into 5 ml of LB-AMP (Sambrook et al., ibid.). The cultures are grown at 37°C until the A_{600} of the culture is approximately 0.5. Isopropyl β-D-thiogalactopyranoside (Sigma) is added to a final concentration of 10 mM to induce production of GFAT, and the culture is allowed to grow at 37°C for two hours. After induction, a 1 ml aliquot is removed from the culture, and the cells are pelleted. The remainder of the culture is allowed to grow for 2 hours after which a 1 ml aliquot is taken, and the cells are pelleted. The pellets were either stored at -20°C before lysis or the pellets are immediately resuspended in 500 µl of 1x sample buffer (0.07 M Tris-HCl (pH 6.8), 0.035% SDS, 10% glycerol, 0.1% bromphenol blue) and boiled for 10 minutes before loading a 25 µl aliquot onto a 10% SDS polyacrylamide gel.

The cell lysates are electrophoresed on a 10% SDS polyacrylamide gel, and the gel is stained with Coomassie blue to visualize the induced GFAT band.

The GFAT activity levels in pPROKGFAT #18 transformants are determined using the method essentially described by Richards and Greengard (Biochim. Biophys Acta 304: 842-850, 1973; which is incorporated by reference herein). Briefly, 50 µl of an overnight culture of XL1-BLUE transformant containing pPROKGFAT #18 is inoculated into 5 ml of LB-AMP (Sambrook et al., ibid.). The cultures are grown and induced as described above. After induction, the cultures are split into five aliquots of 1 ml each. The cells are pelleted, and the cell pellets are frozen at -20°C. The cell pellets subjected to two freeze-thaw cycles, and each pellet is resuspended in 500 µl lysis buffer (100 µM PMSF, 100 mM NaH_2PO_4 (pH 7.5), 50 mM KCl, 10 mM EDTA, 12 mM glucose 6-phosphate). The cells are lysed by sonication on ice. The lysates are centrifuged in a microfuge, and the supernatants, representing the cytosol, are transferred to ice-cold tubes. Total cytosolic protein is determined

using a bicinchoninic acid protein assay kit (Pierce Chemical Co.; Rockford, IL) using the manufacturer's directions.

The supernatants are assayed for GFAT activity using the method essentially described by Richards and Greengard (supra.). The reactions contain 20 µl of 100 mM fructose 6-phosphate (Sigma Chemical Co.), 20 µl of 100 mM glutamine (Sigma Chemical Co.), and 110 µl of a buffer containing 50 mM KCl and 100 mM NaH_2PO_4 (pH 7.5). The reaction is initiated by the addition of 50 µl of cytosol, and the reaction is incubated for 37°C for one hour. The reactions are terminated by the addition of 20 µl of a 50% (vol/vol) ice cold perchloric acid solution. The reaction mixtures are centrifuged for 10 minutes at 4°C in a microfuge. The supernatants are transferred to fresh microfuge tubes, and 25 µl of ice cold 6 N KOH is added to each tube followed by centrifugation at 4°C for 10 minutes in a microfuge.

The glucosamine-6-phosphate content of the supernatant is determined using a modification of the colorimetric method of Levvy and McAllan (Biochemistry 73: 127-132, 1959; which is incorporated by reference herein). Briefly, 150 µl of the supernatant from each sample is added to a 12 x 75 mm glass tubes followed by 100 µl of a saturated tetraborate solution and 10 µl of 1.75% (vol/vol) acetic anhydride in ice cold acetone is added. The reaction mixtures are briefly agitated and placed in a boiling water bath for 4 minutes. After heating, the reactions are placed in an ice water bath for one minute. To each cooled reaction mixture, 200 µl of working solution (Table 1) is added, and the reactions are incubated at 37°C for 20 minutes. After incubation, the reactions are cooled to room temperature, and the absorbance at 585 nm is read.

Table 1

Stock Solution

20 g dimethylaminobenzaldehyde
50 ml concentrated HCl
50 ml glacial acetic acid

The stock solution was stored in an amber bottle at 4°C for up to one month.

Working Solution

1.1 ml Stock solution is diluted to 10 ml with glacial acetic acid.

Glucosamine-6-phosphate is used as a standard, and a unit of GFAT activity is defined as the amount of GFAT that catalyzes the formation of 1 nmole of glucosamine-6-phosphate/min at 37°C.

To demonstrate that the GFAT activity present in the transformed bacteria is due to human GFAT, uridine 5'diphosphate n-acetylglucosamine (UDP-GlcNAc) is added to the reaction mixtures described above. UPD-GlcNAc inhibits human and yeast GFAT, but does not affect the bacterial form of the enzyme. UDP-GlcNAc sensitivity of GFAT activity is assessed in reaction mixtures that are identical with those described above with the exception that the buffer volume as reduced to 100 µl and 10 µl of a 5 mM solution of UDP-GlcNAc (Sigma Chemical Co., St. Louis, MO) was added. The reactions are again initiated by the initiation of 50 µl of cytosol, and the reactions are allowed to proceed for one hour at 37°C. The reactions are terminated, and the levels of glucosamine-6-phosphate are determined as described above.

Example 6 - Anti-GFAT Antibodies

Antisera against recombinant GFAT and a GFAT peptide were raised in New Zealand white rabbits (R and R Rabbitry; Stanwood, CA) and in Balb/c mice (Simonsen Labs; Gilroy, CA).

E. coli transformants containing either pPROK-1 or pPROGFAT were grown, induced and harvested as described above (Example 3). Lysates corresponding to 1-2 ml of cultured, induced cells were thawed, diluted in sample buffer (0.07 M Tris-HCl (pH 6.8), 0.035% SDS, 10% glycerol, 0.1% bromphenol blue) and electrophoresed on a preparative SDS-polyacrylamide gel. The protein was transferred to nitrocellulose (Schleicher & Schuell; Keene, NH) in 1x Transfer Buffer (Table 2) in a Bio-Rad TRANSBLOT (BioRad Laboratories; Richmond, CA) at 400 milliamps for ninety minutes at 4°C. The nitrocellulose was rinsed in distilled water and stained for five minutes in Ponceau S (Sigma; 0.1% w/v in 1% acetic acid). The nitrocellulose was destained in distilled water. The 77 kDa GFAT band was identified and cut out with a clean razor blade. The nitrocellulose band was destained completely in water and dried. This procedure generated enough protein for the immunization of five mice.

Table 2

1x Transfer Buffer
1.1 M Tris
1.2 M Glycine
0.1% SDS
20% Methanol

1x RIPA
10 mM Tris (pH 7.4)
150 mM NaCl
1% sodium deoxycholate
1% NP-40
0.1% SDS

Wash Buffer 1
1 M NaCl
1.3 M Tris (pH 7.5)
0.1% NP-40

Wash Buffer 2
1.4 M NaCl
1.5 M Tris (pH 7.5)
1% NP-40
0.5% Triton

Wash Buffer 3
1.6 M Tris (pH 7.5)
0.1% NP-40

A synthetic peptide having the sequence of Sequence ID NO: 2 from Cysteine, amino acid number 493, to Glutamic acid, amino acid number 505, and containing a C-terminal Tyrosine residue to facilitate conjugation was synthesized on an Applied Biosystems Model 431A peptide synthesizer (Foster City, CA) using standard cycles as

directed by the manufacturer and Fmoc chemistry essentially as described by Carpino and Han (J. Amer. Chem. Soc. 92: 5748-5749, 1970; J. Org. Chem. 37: 3404-3409, 1972). An unloaded HMP (p-alkyloxybenzyl alcohol) resin was used. The first amino acid was coupled to the resin as a symmetric anhydride. Subsequent amino acids were coupled as HBTU/HOBt active esters. When the synthesis was complete, the final Fmoc protecting group was removed and the resin was dried.

During synthesis, a resin sample was taken after each coupling. Samples were assayed as directed by the manufacturer. The first sample was used to test the efficiency of the resin loading, which was found to be 100%. The efficiency of coupling was assayed using a ninhydrin assay. The coupling efficiency was found to be greater than 99% for all coupling reactions.

The peptide was cleaved from the resin using 95% trifluoroacetic acid (TFA). The peptide was precipitated in diethyl ether and redissolved in 10% acetic acid. The peptide was purified on a reverse-phase HPLC using a C-4 column with a H_2O/acetonitrile (both containing 0.1% TFA) gradient. The main peak was collected, a sample was taken for amino acid analysis and the peptide was lyophilized.

The peptide was coupled to KLH activated maleimide (Chemicon; Temecula, CA) for use as an immunogen. Ten milligrams of the peptide was diluted in 400 µl of 10 mM HAc. A reaction mixture containing the peptide, 5.6 ml phosphate buffered saline (PBS; Sigma Chemical Co., St. Louis, MO) and 1 ml of a 10 mg/ml KLH activated maleimide solution was rocked at 4°C for 22 hours. The resulting mixture was aliquotted into 24 vials containing approximately 410 µg of conjugated peptide per vial. Two rabbits were injected subcutaneously with 205 µg of conjugated peptide each every three weeks. Five female Balb/C mice were injected

intraperitoneally with 82 μg of conjugated peptide each every two weeks for a total of six injections.

The nitrocellulose band containing GFAT (prepared as described above) was dissolved in a minimal amount of DMSO using between 250 μl and 375 μl of DMSO, but not more than 500 μl of DMSO. The DMSO-solubilized nitrocellulose bands were divided into five equal aliquots. Five young female Balb/c mice were injected intraperitoneally with the DMSO-solubilized nitrocellulose bands of GFAT. The injections were repeated every two weeks for a total of six injections.

Sera from the immunized animals were tested for the ability to immunoprecipitate recombinant GFAT. E. coli strain XL1-BLUE (Stratagene) transformed with either pPROGFAT or pPROK-1 (negative control) were grown overnight at 37°C in LB media (Sambrook et al., ibid.) supplemented with 100 μg ampicillin/ml. The cultures were diluted 1:40 into 5 ml of LB media, and the cultures were grown for 2.5 hours at 37°C. The cells were pelleted, and the cell pellets were resuspended in 5 ml M9 media (Sambrook et al., ibid.). Each culture received IPTG to a final concentration of 10 mM, and the cultures were grown for 2 hours at 37°C. After the 2 hour period, 25 μCi/ml of ^{35}S-EXPRESS (NEN; Boston, MA) was added to each culture, and the cultures were incubated for 10 minutes at 37°C. After incubation, the cells were centrifuged, and the cell pellets were washed twice with PBS. The volume of the cell pellets were approximated, and 10 volumes of a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0) and 4 mg/ml lysozyme was added to each pellet to lyse the cells. The resuspended pellets were incubated for 5 minutes at room temperature after which 500 μl of 1x RIPA (Table 2) was added to each lysate. The lysates were incubated on ice for 30 minutes followed by centrifugation in a microfuge for 15 minutes at 4°C. The supernatants were transferred to fresh tubes and were stored at -70°C.

Before use, the lysates were thawed on ice and then centrifuged in a microfuge for 15 minutes to obtain clarified supernatants. To confirm induction of human GFAT production, a 30 µl aliquot of each clarified lysate was electrophoresed on an SDS-polyacrylamide gel, and the gel was exposed to film. The lysates were then immunoprecipitated with the rabbit and mouse polyclonal antibodies. For each reaction, 100 µl of lysate was combined with 100-fold diluted antisera of either the rabbit anti-peptide polyclonal, the mouse anti-peptide polyclonal or the mouse anti-recombinant GFAT polyclonal, and the reactions were incubated on ice for 60 minutes. For immunoprecipitation reactions using mouse polyclonal antibodies, 5 µl of rabbit anti-mouse polyclonal antibodies (Sigma) was added to each reaction mixture, and the mixtures were incubated for 30 minutes on ice. After the 60 minute incubation, 100 µl of Staph A (PANSORBIN; Calbiochem; San Diego, CA) that had been prewashed with 1x RIPA (Table 2) was added to each reaction tube, and the reactions were incubated on ice for 30 minutes. The reactions were pelleted, and the pellets were subjected to 500 µl sequential washes with Wash Buffer 1 (Table 2), Wash Buffer 2 (Table 2), and then Wash Buffer 3 (Table 2). After the wash with Wash Buffer 3 (Table 2), the reactions were centrifuged. The supernatants were discarded, the pellets were resuspended in 50 µl of 1x Sample Buffer, and the samples were boiled for 10 minutes at 95°C. The boiled samples were subjected to SDS-polyacrylamide gel electrophoresis, and the gel was fixed in 10% HAc, 20% methanol for 30 minutes. After fixing, the gel was soaked in AMPLIFY (Amersham; Arlington Hts., IL) for 20 minutes. The gel was dried and exposed to film with a screen at -70°C. The presence of bands at 77 kDa indicated that each antisera was capable of immunoprecipitating recombinant human GFAT. Anti-GFAT peptide rabbit antisera is

affinity purified on a GFAT peptide column. Affinity purified antisera is used in methods for purifying GFAT.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

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Smith, Robert A
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(ii) TITLE OF INVENTION: GLUTAMINE FRUCTOSE-6-PHOSPHATE AMIDOTRANSFERASE

(iii) NUMBER OF SEQUENCES: 19

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3089 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(A) NAME/KEY: CDS
(B) LOCATION: 123..2165

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AGGGAGTCGT GTCGGCGCCA CCCCGGCCCC CGAGCCCGCA GATTGCCCAC CGAAGCTCGT      60

GTGTGCACCC CCGATCCCGC CAGCCACTCG CCCCTGGCCT CGCGGGCCGT GTCTCCGGCA     120

TC ATG TGT GGT ATA TTT GCT TAC TTA AAC TAC CAT GTT CCT CGA ACG         167
   Met Cys Gly Ile Phe Ala Tyr Leu Asn Tyr His Val Pro Arg Thr
     1               5                  10                  15

AGA CGA GAA ATC CTG GAG ACC CTA ATC AAA GGC CTT CAG AGA CTG GAG       215
Arg Arg Glu Ile Leu Glu Thr Leu Ile Lys Gly Leu Gln Arg Leu Glu
                20                  25                  30
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TAC AGA GGA TAT GAT TCT GCT GGT GTG GGA TTT GAT GGA GGC AAT GAT      263
Tyr Arg Gly Tyr Asp Ser Ala Gly Val Gly Phe Asp Gly Gly Asn Asp
            35                  40                  45

AAA GAT TGG GAA GCC AAT GCC TGC AAA ACC CAG CTT ATT AAG AAG AAA      311
Lys Asp Trp Glu Ala Asn Ala Cys Lys Thr Gln Leu Ile Lys Lys Lys
        50                  55                  60

GGA AAA GTT AAG GCA CTG GAT GAA GAA GTT CAC AAG CAA CAA GAT ATG      359
Gly Lys Val Lys Ala Leu Asp Glu Glu Val His Lys Gln Gln Asp Met
    65                  70                  75

GAT TTG GAT ATA GAA TTT GAT GTA CAC CTT GGA ATA GCT CAT ACC CGT      407
Asp Leu Asp Ile Glu Phe Asp Val His Leu Gly Ile Ala His Thr Arg
80                  85                  90                  95

TGG GCA ACA CAT GGA GAA CCC AGT CCT GTC AAT AGC CAC CCC CAG CGC      455
Trp Ala Thr His Gly Glu Pro Ser Pro Val Asn Ser His Pro Gln Arg
                100                 105                 110

TCT GAT AAA AAT AAT GAA TTT ATC GTT ATT CAC AAT GGA ATC ATC ACC      503
Ser Asp Lys Asn Asn Glu Phe Ile Val Ile His Asn Gly Ile Ile Thr
            115                 120                 125

AAC TAC AAA GAC TTG AAA AAG TTT TTG GAA AGC AAA GGC TAT GAC TTC      551
Asn Tyr Lys Asp Leu Lys Lys Phe Leu Glu Ser Lys Gly Tyr Asp Phe
        130                 135                 140

GAA TCT GAA ACA GAC ACA GAG ACA ATT GCC AAG CTC GTT AAG TAT ATG      599
Glu Ser Glu Thr Asp Thr Glu Thr Ile Ala Lys Leu Val Lys Tyr Met
    145                 150                 155

TAT GAC AAT CGG GAA AGT CAA GAT ACC AGC TTT ACT ACC TTG GTG GAG      647
Tyr Asp Asn Arg Glu Ser Gln Asp Thr Ser Phe Thr Thr Leu Val Glu
160                 165                 170                 175

AGA GTT ATC CAA CAA TTG GAA GGT GCT TTT GCA CTT GTG TTT AAA AGT      695
Arg Val Ile Gln Gln Leu Glu Gly Ala Phe Ala Leu Val Phe Lys Ser
                180                 185                 190

GTT CAT TTT CCC GGG CAA GCA GTT GGC ACA AGG CGA GGT AGC CCT CTG      743
Val His Phe Pro Gly Gln Ala Val Gly Thr Arg Arg Gly Ser Pro Leu
            195                 200                 205
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TTG ATT GGT GTA CGG AGT GAA CAT AAA CTT TCT ACT GAT CAC ATT CCT        791
Leu Ile Gly Val Arg Ser Glu His Lys Leu Ser Thr Asp His Ile Pro
        210                 215                 220

ATA CTC TAC AGA ACA GGC AAA GAC AAG AAA GGA AGC TGC AAT CTC TCT        839
Ile Leu Tyr Arg Thr Gly Lys Asp Lys Lys Gly Ser Cys Asn Leu Ser
    225                 230                 235

CGT GTG GAC AGC ACA ACC TGC CTT TTC CCG GTG GAA GAA AAA GCA GTG        887
Arg Val Asp Ser Thr Thr Cys Leu Phe Pro Val Glu Glu Lys Ala Val
240                 245                 250                 255

GAG TAT TAC TTT GCT TCT GAT GCA AGT GCT GTC ATA GAA CAC ACC AAT        935
Glu Tyr Tyr Phe Ala Ser Asp Ala Ser Ala Val Ile Glu His Thr Asn
                260                 265                 270

CGC GTC ATC TTT CTG GAA GAT GAT GAT GTT GCA GCA GTA GTG GAT GGA        983
Arg Val Ile Phe Leu Glu Asp Asp Asp Val Ala Ala Val Val Asp Gly
            275                 280                 285

CGT CTT TCT ATC CAT CGA ATT AAA CGA ACT GCA GGA GAT CAC CCC GGA       1031
Arg Leu Ser Ile His Arg Ile Lys Arg Thr Ala Gly Asp His Pro Gly
        290                 295                 300

CGA GCT GTG CAA ACA CTC CAG ATG GAA CTC CAG CAG ATC ATG AAG GGC       1079
Arg Ala Val Gln Thr Leu Gln Met Glu Leu Gln Gln Ile Met Lys Gly
    305                 310                 315

AAC TTC AGT TCA TTT ATG CAG AAG GAA ATA TTT GAG CAG CCA GAG TCT       1127
Asn Phe Ser Ser Phe Met Gln Lys Glu Ile Phe Glu Gln Pro Glu Ser
320                 325                 330                 335

GTC GTG AAC ACA ATG AGA GGA AGA GTC AAC TTT GAT GAC TAT ACT GTG       1175
Val Val Asn Thr Met Arg Gly Arg Val Asn Phe Asp Asp Tyr Thr Val
                340                 345                 350

AAT TTG GGT GGT TTG AAG GAT CAC ATA AAG GAG ATC CAG AGA TGC CGG       1223
Asn Leu Gly Gly Leu Lys Asp His Ile Lys Glu Ile Gln Arg Cys Arg
            355                 360                 365

CGT TTG ATT CTT ATT GCT TGT GGA ACA AGT TAC CAT GCT GGT GTA GCA       1271
Arg Leu Ile Leu Ile Ala Cys Gly Thr Ser Tyr His Ala Gly Val Ala
        370                 375                 380
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ACA CGT CAA GTT CTT GAG GAG CTG ACT GAG TTG CCT GTG ATG GTG GAA      1319
Thr Arg Gln Val Leu Glu Glu Leu Thr Glu Leu Pro Val Met Val Glu
    385                 390                 395

CTA GCA AGT GAC TTC CTG GAC AGA AAC ACA CCA GTC TTT CGA GAT GAT      1367
Leu Ala Ser Asp Phe Leu Asp Arg Asn Thr Pro Val Phe Arg Asp Asp
400                 405                 410                 415

GTT TGC TTT TTC CTT AGT CAA TCA GGT GAG ACA GCA GAT ACT TTG ATG      1415
Val Cys Phe Phe Leu Ser Gln Ser Gly Glu Thr Ala Asp Thr Leu Met
                420                 425                 430

GGT CTT CGT TAC TGT AAG GAG AGA GGA GCT TTA ACT GTG GGG ATC ACA      1463
Gly Leu Arg Tyr Cys Lys Glu Arg Gly Ala Leu Thr Val Gly Ile Thr
            435                 440                 445

AAC ACA GTT GGC AGT TCC ATA TCA CGG GAG ACA GAT TGT GGA GTT CAT      1511
Asn Thr Val Gly Ser Ser Ile Ser Arg Glu Thr Asp Cys Gly Val His
        450                 455                 460

ATT AAT GCT GGT CCT GAG ATT GGT GTG GCC AGT ACA AAG GCT TAT ACC      1559
Ile Asn Ala Gly Pro Glu Ile Gly Val Ala Ser Thr Lys Ala Tyr Thr
    465                 470                 475

AGC CAG TTT GTA TCC CTT GTG ATG TTT GCC CTT ATG ATG TGT GAT GAT      1607
Ser Gln Phe Val Ser Leu Val Met Phe Ala Leu Met Met Cys Asp Asp
480                 485                 490                 495

CGG ATC TCC ATG CAA GAA AGA CGC AAA GAG ATC ATG CTT GGA TTG AAA      1655
Arg Ile Ser Met Gln Glu Arg Arg Lys Glu Ile Met Leu Gly Leu Lys
                500                 505                 510

CGG CTG CCT GAT TTG ATT AAG GAA GTA CTG AGC ATG GAT GAC GAA ATT      1703
Arg Leu Pro Asp Leu Ile Lys Glu Val Leu Ser Met Asp Asp Glu Ile
            515                 520                 525

CAG AAA CTA GCA ACA GAA CTT TAT CAT CAG AAG TCA GTT CTG ATA ATG      1751
Gln Lys Leu Ala Thr Glu Leu Tyr His Gln Lys Ser Val Leu Ile Met
        530                 535                 540

GGA CGA GGC TAT CAT TAT GCT ACT TGT CTT GAA GGG GCA CTG AAA ATC      1799
Gly Arg Gly Tyr His Tyr Ala Thr Cys Leu Glu Gly Ala Leu Lys Ile
    545                 550                 555
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AAA GAA ATT ACT TAT ATG CAC TCT GAA GGC ATC CTT GCT GGT GAA TTG      1847
Lys Glu Ile Thr Tyr Met His Ser Glu Gly Ile Leu Ala Gly Glu Leu
560                 565                 570                 575

AAA CAT GGC CCT CTG GCT TTG GTG GAT AAA TTG ATG CCT GTG ATC ATG      1895
Lys His Gly Pro Leu Ala Leu Val Asp Lys Leu Met Pro Val Ile Met
                580                 585                 590

ATC ATC ATG AGA GAT CAC ACT TAT GCC AAG TGT CAG AAT GCT CTT CAG      1943
Ile Ile Met Arg Asp His Thr Tyr Ala Lys Cys Gln Asn Ala Leu Gln
            595                 600                 605

CAA GTG GTT GCT CGG CAG GGG CGG CCT GTG GTA ATT TGT GAT AAG GAG      1991
Gln Val Val Ala Arg Gln Gly Arg Pro Val Val Ile Cys Asp Lys Glu
        610                 615                 620

GAT ACT GAG ACC ATT AAG AAC ACA AAA AGA ACG ATC AAG GTG CCC CAC      2039
Asp Thr Glu Thr Ile Lys Asn Thr Lys Arg Thr Ile Lys Val Pro His
    625                 630                 635

TCA GTG GAC TGC TTG CAG GGC ATT CTC AGC GTG ATC CCT TTA CAG TTG      2087
Ser Val Asp Cys Leu Gln Gly Ile Leu Ser Val Ile Pro Leu Gln Leu
640                 645                 650                 655

CTG GCT TTC CAC CTT GCT GTG CTG AGA GGC TAT GAT GTT GAT TTC CCA      2135
Leu Ala Phe His Leu Ala Val Leu Arg Gly Tyr Asp Val Asp Phe Pro
                660                 665                 670

CGG AAT CTT GCC AAA TCT GTG ACT GTA GAG TGAGGAATAT CTATACAAAA        2185
Arg Asn Leu Ala Lys Ser Val Thr Val Glu
            675                 680

TGTACGAAAC TGTATGATTA AGCAACACAA GACACCTTTT GTATTTAAAA CCTTGATTTA    2245

AAATATCACC CCTTGAAGCC TTTTTTTAGT AAATCCTTAT TTATATATCA GTTATAATTA    2305

TTCCACTCAA TATGTGATTT TTGTGAAGTT ACCTCTTACA TTTTCCCAGT AATTTGTGGA    2365

GGACTTTGAA TAATGGAATC TATATTGGAA TCTGTATCAG AAAGATTCTA GCTATTATTT    2425

TCTTTAAAGA ATGCTGGGTG TTGCATTTCT GGACCCTCCA CTTCAATCTG AGAAGACAAT    2485

ATGTTTCTAA AAATTGGTAC TTGTTTCACC ATACTTCATT CAGACCAGTG AAAGAGTAGT    2545

GCATTTAATT GGAGTATCTA AAGCCAGTGG CAGTGTATGC TCATACTTGG ACAGTTAGGG    2605
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AAGGGTTTGC CAAGTTTTAA GAGAAGATGT GATTTATTTT GAAATTTGTT TCTGTTTTGT 2665

TTTTAAATCA AACTGTAAAA CTTAAAACTG AAAAATTTTA TTGGTAGGAT TTATATCTAA 2725

GTTTGGTTAG CCTTAGTTTC TCAGACTTGT TGTCTATTAT CTGTAGGTGG AAGAAATTTA 2785

GGAAGCGAAA TATTACAGTA GTGCATTGGT GGGTCTCAAT CCTTAACATA TTTGCACAAT 2845

TTTATAGCAC AAACTTTAAA TTCAAGCTGC TTTGGACAAC TGACAATATG ATTTTAAATT 2905

TGAAGATGGG ATGTGTACAT GTTGGGTATC CTACTACTTT GTGTTTTCAT CTCCTAAAAG 2965

TGTTTTTTAT TTCCTTGTAT CTGTAGTCTT TTATTTTTTA AATGACTGCT GAATGACATA 3025

TTTTATCTTG TTCTTTAAAA TCACAACACA GAGCTGCTAT TAAATTAATA TTGATATAAA 3085

AAAA 3089

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 681 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Cys Gly Ile Phe Ala Tyr Leu Asn Tyr His Val Pro Arg Thr Arg
1 5 10 15

Arg Glu Ile Leu Glu Thr Leu Ile Lys Gly Leu Gln Arg Leu Glu Tyr
20 25 30

Arg Gly Tyr Asp Ser Ala Gly Val Gly Phe Asp Gly Gly Asn Asp Lys
35 40 45

Asp Trp Glu Ala Asn Ala Cys Lys Thr Gln Leu Ile Lys Lys Lys Gly
50 55 60

Lys Val Lys Ala Leu Asp Glu Glu Val His Lys Gln Gln Asp Met Asp
65 70 75 80

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Leu Asp Ile Glu Phe Asp Val His Leu Gly Ile Ala His Thr Arg Trp
                85                  90                  95

Ala Thr His Gly Glu Pro Ser Pro Val Asn Ser His Pro Gln Arg Ser
            100                 105                 110

Asp Lys Asn Asn Glu Phe Ile Val Ile His Asn Gly Ile Ile Thr Asn
        115                 120                 125

Tyr Lys Asp Leu Lys Lys Phe Leu Glu Ser Lys Gly Tyr Asp Phe Glu
    130                 135                 140

Ser Glu Thr Asp Thr Glu Thr Ile Ala Lys Leu Val Lys Tyr Met Tyr
145                 150                 155                 160

Asp Asn Arg Glu Ser Gln Asp Thr Ser Phe Thr Thr Leu Val Glu Arg
                165                 170                 175

Val Ile Gln Gln Leu Glu Gly Ala Phe Ala Leu Val Phe Lys Ser Val
            180                 185                 190

His Phe Pro Gly Gln Ala Val Gly Thr Arg Arg Gly Ser Pro Leu Leu
        195                 200                 205

Ile Gly Val Arg Ser Glu His Lys Leu Ser Thr Asp His Ile Pro Ile
    210                 215                 220

Leu Tyr Arg Thr Gly Lys Asp Lys Lys Gly Ser Cys Asn Leu Ser Arg
225                 230                 235                 240

Val Asp Ser Thr Thr Cys Leu Phe Pro Val Glu Glu Lys Ala Val Glu
                245                 250                 255

Tyr Tyr Phe Ala Ser Asp Ala Ser Ala Val Ile Glu His Thr Asn Arg
            260                 265                 270

Val Ile Phe Leu Glu Asp Asp Asp Val Ala Ala Val Val Asp Gly Arg
        275                 280                 285

Leu Ser Ile His Arg Ile Lys Arg Thr Ala Gly Asp His Pro Gly Arg
    290                 295                 300

Ala Val Gln Thr Leu Gln Met Glu Leu Gln Gln Ile Met Lys Gly Asn
305                 310                 315                 320
```

Phe Ser Ser Phe Met Gln Lys Glu Ile Phe Glu Gln Pro Glu Ser Val
325 330 335

Val Asn Thr Met Arg Gly Arg Val Asn Phe Asp Asp Tyr Thr Val Asn
340 345 350

Leu Gly Gly Leu Lys Asp His Ile Lys Glu Ile Gln Arg Cys Arg Arg
355 360 365

Leu Ile Leu Ile Ala Cys Gly Thr Ser Tyr His Ala Gly Val Ala Thr
370 375 380

Arg Gln Val Leu Glu Glu Leu Thr Glu Leu Pro Val Met Val Glu Leu
385 390 395 400

Ala Ser Asp Phe Leu Asp Arg Asn Thr Pro Val Phe Arg Asp Asp Val
405 410 415

Cys Phe Phe Leu Ser Gln Ser Gly Glu Thr Ala Asp Thr Leu Met Gly
420 425 430

Leu Arg Tyr Cys Lys Glu Arg Gly Ala Leu Thr Val Gly Ile Thr Asn
435 440 445

Thr Val Gly Ser Ser Ile Ser Arg Glu Thr Asp Cys Gly Val His Ile
450 455 460

Asn Ala Gly Pro Glu Ile Gly Val Ala Ser Thr Lys Ala Tyr Thr Ser
465 470 475 480

Gln Phe Val Ser Leu Val Met Phe Ala Leu Met Met Cys Asp Asp Arg
485 490 495

Ile Ser Met Gln Glu Arg Arg Lys Glu Ile Met Leu Gly Leu Lys Arg
500 505 510

Leu Pro Asp Leu Ile Lys Glu Val Leu Ser Met Asp Asp Glu Ile Gln
515 520 525

Lys Leu Ala Thr Glu Leu Tyr His Gln Lys Ser Val Leu Ile Met Gly
530 535 540

Arg Gly Tyr His Tyr Ala Thr Cys Leu Glu Gly Ala Leu Lys Ile Lys
545 550 555 560

Glu Ile Thr Tyr Met His Ser Glu Gly Ile Leu Ala Gly Glu Leu Lys
565 570 575

His Gly Pro Leu Ala Leu Val Asp Lys Leu Met Pro Val Ile Met Ile
580 585 590

Ile Met Arg Asp His Thr Tyr Ala Lys Cys Gln Asn Ala Leu Gln Gln
595 600 605

Val Val Ala Arg Gln Gly Arg Pro Val Val Ile Cys Asp Lys Glu Asp
610 615 620

Thr Glu Thr Ile Lys Asn Thr Lys Arg Thr Ile Lys Val Pro His Ser
625 630 635 640

Val Asp Cys Leu Gln Gly Ile Leu Ser Val Ile Pro Leu Gln Leu Leu
645 650 655

Ala Phe His Leu Ala Val Leu Arg Gly Tyr Asp Val Asp Phe Pro Arg
660 665 670

Asn Leu Ala Lys Ser Val Thr Val Glu
675 680

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2487

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACTCGAGTC GACATCGATC AGTTTTTTTT TTTTTTTTT 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3866

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGNMCNGARA THGGNGTNGC 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3867

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCTAGAATTC GGNMCNGARA THGGNGTNGC 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3868

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCRTGYTTNA RYTCNCCNGC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3869

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATGGATCC CCRTGYTTNA RYTCNCCNGC 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4306

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTACAAAGGC TTATACCAGC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4307

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGATGCCTTC AGAGTGC 17

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4764

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACGTCCATC CACTACTGCT 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4804

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAGGCAATG ATAAAGATTG 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4839

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGAATTCATG CCATGTGTGG TATATTTGCT 30

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4866

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAAATTCCT ATCACTCTAC AGTCACAGAT T 31

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4949

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTAGCGTCTG 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4950

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTCAGACG 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2435

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATCGACCGG ATCGGAAAAC C 21

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC5192

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTNGCYTCCC ARTCYTTRTC RTT 23

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC6089

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGGAATTCAT GTGTGGTATA TTTG 24

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC6090

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/973,330
(I) FILING DATE: 05-NOV-1992
(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: US 07/872,648
(I) FILING DATE: 22-NOV-1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTTTCTTGT CTTTGCCTGT TCT 23

Claims

We claim:

1. An isolated human glutamine:fructose-6-phosphate amido-transferase.

2. An isolated human glutamine:fructose-6-phosphate amido-transferase according to claim 1, which has the amino acid sequence of Sequence ID NO: 2 from methionine, amino acid number 1 to glutamic acid, amino acid number 681.

3. An antiserum obtained from an animal immunized with the glutamine:fructose-6-phosphate amido-transferase of claim 1 wherein said antiserum binds to human glutamine:fructose-6-phosphate amido-transferase.

4. A monoclonal antibody which binds to the glutamine:fructose-6-phosphate amido-transferase of claim 1.

5. An isolated DNA molecule encoding human glutamine:fructose-6-phosphate amido-transferase.

6. An isolated DNA molecule according to claim 5, wherein said molecule comprises the nucleotide sequence shown in Sequence ID NO: 1 from nucleotide 123 to nucleotide 2165.

7. An isolated DNA molecule according to claim 5, which encodes the amino acid sequence of Sequence ID NO: 2 from methionine, amino acid number 1, to glutamic acid, amino acid number 681.

8. A DNA molecule of at least about 14 nucleotides, wherein said molecule is capable of hybridizing with a gene which encodes a human glutamine:fructose-6-phosphate amidotransferase polypeptide, and wherein said DNA molecule is at least 85% homologous to a corresponding DNA sequence of the human glutamine:fructose-6-phosphate amidotransferase of Sequence ID NO: 1 or its complement.

9. A DNA molecule according to claim 8, wherein said molecule is labeled to provide a detectable signal.

10. A DNA construct capable of expressing an antisense RNA molecule comprising the following operably linked elements:

a transcriptional promoter;

a DNA molecule of at least about 14 nucleotides, wherein said molecule is capable of hybridizing with a gene which encodes a human glutamine:fructose-6-phosphate amidotransferase polypeptide, and wherein said DNA molecule is at least 85% homologous to a corresponding DNA sequence of the human glutamine:fructose-6-phosphate amidotransferase of Sequence ID NO: 1 or its complement; and

a transcriptional terminator.

11. A DNA construct comprising the following operably linked elements:

a transcriptional promoter;

a DNA molecule encoding a human glutamine:fructose-6-phosphate amido-transferase; and

a transcriptional terminator.

12. A DNA construct according to claim 11, wherein the DNA molecule comprises the nucleotide sequence of Sequence ID NO: 1 from nucleotide 123 to nucleotide 2165.

13. A DNA construct according to claim 11, wherein the DNA molecule encodes the amino acid sequence of Sequence ID NO: 2 from methionine, amino acid number 1, to glutamic acid, amino acid number 681.

14. A host cell transformed or transfected with a DNA construct, wherein the construct comprises the following operably linked elements:

a transcriptional promoter;

a DNA molecule encoding a human glutamine:fructose-6-phosphate amido-transferase; and

a transcriptional terminator.

15. A host cell according to claim 14, wherein the DNA molecule comprises the nucleotide sequence of the Figure (Sequence ID NO: 1) from nucleotide 123 to nucleotide 2165.

16. A host cell according to claim 14, wherein the DNA molecule encodes the amino acid sequence of the Figure (Sequence ID NO: 2) from methionine, amino acid number 1, to glutamic acid, amino acid number 681.

17. A host cell according to claim 14, wherein said host cell is a cultured mammalian cell, a fungal cell or a bacterial cell.

18. A method for producing a human glutamine:fructose-6-phosphate amido-transferase, which comprises:

growing host cells transformed or transfected with an expression vector which comprises a DNA molecule encoding a human glutamine:fructose-6-phosphate amido-transferase under suitable conditions to allow the expression of human glutamine:fructose-6-phosphate amidotransferase encoded by said DNA molecule, and

isolating the glutamine:fructose-6-phosphate amido-transferase from the cells.

19. A method according to claim 18, wherein the cells are cultured mammalian cells, bacterial cells or fungal cells.

20. A method according to claim 18, wherein the DNA molecule comprises the nucleotide sequence of Sequence ID NO: 1 from nucleotide 123 to nucleotide 2165.

21. A method according to claim 18, wherein the DNA molecule encodes the amino acid sequence of Sequence ID NO: 2 from methionine, amino acid number 1, to glutamic acid, amino acid number 681.

22. A method for detecting a compound which inhibits human glutamine:fructose-6-phosphate amido-transferase comprising the steps of:

a) exposing a test substance to human glutamine:fructose-6-phosphate amido-transferase in the presence of fructose-6-phosphate and glutamine under physiological conditions and for a time sufficient to allow the test substance to inhibit glutamine:fructose-6-phosphate amidotransferase activity; and

b) detecting a reduction in activity of the glutamine:fructose-6-phosphate amido-transferase in comparison to the activity in the absence of the test substance and therefrom determining the presence in the test substance of a compound which inhibits human glutamine:fructose-6-phosphate amido-transferase.

23. A method according to claim 22, wherein said step of exposing comprises combining a test substance with human glutamine:fructose-6-phosphate amido-transferase in the presence of fructose-6-phosphate and radiolabeled glutamine and the step of detecting comprises measuring the production of radiolabeled glutamate relative to the production of radiolabeled glutamate in the absence of the test substance.

24. A method according to claim 22 wherein said step of exposing comprises exposing a test substance to human glutamine:fructose-6-phosphate amido-transferase in the presence of 3-acetylpyridine adenine dinucleotide, glutamate dehydrogenase, fructose-6-phosphate and glutamine, and the step of detecting comprises measuring 3-acetylpyridine adenine dinucleotide production relative to the production of 3-

acetylpyridine adenine dinucleotide in the absence of the test substance.

25. A method according to claim 22 wherein said glutamine:fructose-6-phosphate amidotransferase is recombinant glutamine:fructose-6-phosphate amidotransferase.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/03773

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/54; C12N9/10; C12N5/10; C12P21/08
C12N15/11; C12Q1/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X,P	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 267, no. 35, 15 December 1992, BALTIMORE, MD US pages 25208 - 25212 MCKNIGHT, G.L. ET AL. 'Molecular cloning, cDNA sequence, and bacterial expression of human glutamine;fructose-6-phosphate amidotransferase' see the whole document --- -/--	1-25

[10] Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 AUGUST 1993	23 -09- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 242, no. 13, 10 July 1967, BALTIMORE, MD US pages 3135 - 3141 KORNFELD, R. 'Studies on L-glutamine D-fructose 6-phosphate Amidotransferase'	1,22
Y	see page 3136, column 2, paragraph 1 see page 3137, column 1, paragraph 3; figures 1,2,3; table II	2-21, 23-25
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 264, no. 15, 25 May 1989, BALTIMORE, MD US pages 8753 - 8758 WATZELE, G. ET TANNER, W. 'Cloning of the glutamine:fructose-6-phosphate amidotransferase gene from yeast' cited in the application see the whole document	2-21, 23-25
Y	BIOCHEMISTRY. vol. 26, 1987, EASTON, PA US pages 1940 - 1948 BADET,B. ET AL. 'Glucosamine synthetase from Escherichia coli: purification, properties, and glutamine-utilizing site location' cited in the application see the whole document	2-21, 23-25
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 266, no. 16, 5 June 1991, BALTIMORE, MD US pages 10155 - 10161 MARSHALL, S. ET AL. 'Complete inhibition of glucose-induced desensitization of the glucose transport system by inhibitors of mRNAs synthesis' cited in the application see the whole document	1

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 266, no. 16, 5 June 1991, BALTIMORE, MD US pages 10148 - 10154 TRAXINGER, R.R. ET MARSHALL, S. 'Coordinated regulation of glutamine:fructose-6-phosphate amidotransferase activity by insulin, glucose, and glutamine' cited in the application see the whole document	1
A	THE INTERNATIONAL JOURNAL OF BIOCHEMISTRY vol. 5, 1974, pages 515 - 523 TRUJILLO, J. L. & GAN, J.C. 'Purification and some kinetic properties of bovine thyroid gland L-glutamine : D-fructose-6-phosphate amidotransferase' see the whole document	1
A	BIOCHEMICAL JOURNAL vol. 121, no. 4, 1971, pages 701 - 709 WINTERBURN, P. J. & PHELPS, C.F. 'Purification and some kinetic properties of rat liver glucosamine synthetase' see the whole document	1